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PRINCIPAL INVESTIGATOR: Amy Brooks-Kayal, M.D.

CONTRACTING ORGANIZATION: University of Colorado Denver School of Medicine
Aurora, CO 80045

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Amy Brooks-Kayal, M.D

E-Mail: amy.brooks-kayal@childrenscolorado.org

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14. ABSTRACT

Our proposal tests the hypothesis that JaK/ATAT pathway activation after TBI leads to Gabra1 repression and is a critical mediator of post-traumatic epileptogenesis and epilepsy progression, and that inhibition of this pathway at the time of TBI and/or after development of post-traumatic epilepsy will inhibit epilepsy development and/or progression after CCI. In the first year of funding, equipment and training necessary to perform the CCI model was obtained at University of Colorado, and CCI was successfully performed in mice at both institutions. Training was completed for all personnel on all necessary molecular, anatomical, electrophysiological and neurophysiological (EEG) techniques, essential baseline data was obtained in the mouse CCI model and specific outcome measures were established. Moreover, issues with the JAK/STAT3 inhibitor (WP1066) have been mitigated and the drug is now successfully used in all labs. Additional optimization of the WP1066 dosing protocol is underway and is expected to be completed very soon. Two manuscripts and the three abstracts related to GABA(A) receptor, JAK/STAT pathway and cell death alterations following TBI were published during the funding period..

15. SUBJECT TERMS- CCI-controlled cortical impact; TBI-traumatic brain injury; Gabra1- GABA(A) Receptor subunit gene; JaK/STAT – Janus Kinase/Signal Transducer and Activator of Transcription.

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INTRODUCTION:

Our overall hypothesis is that JaK/STAT pathway activation after TBI leads to GABA(A) receptor α1 subunit gene (*Gabra1*) repression and is a critical mediator of post-traumatic epileptogenesis and epilepsy progression. The JaK/STAT pathway has not been studied in post-traumatic epilepsy, but beyond its role in *Gabra1* regulation, it is known to be an important regulator of neuronal proliferation, survival and gliogenesis, all of which may be important contributors to epileptogenesis. The Specific Aims of our study are:

1. Determine whether activation of the JaK/STAT pathway and downregulation of GABA(A) Receptor **α**1 subunit gene (*Gabra1*) transcription occur following traumatic brain injury (TBI) and subsequent epileptogenesis. We hypothesize that activation of the JaK/STAT pathway, downregulation of Gabra1 and subsequent reduction of **α**1 subunit levels occur in injured hippocampus and cortex following TBI and contribute to post-traumatic epileptogenesis. To test this hypothesis, we will examine activation of the JaK/STAT pathway, and levels of ICER and $GABA_AR$ subunits (Gabr) acutely and chronically in the controlled cortical impact (CCI) model in mice before and after development of spontaneous seizures

2. Determine whether activation of the JaK/STAT pathway and downregulation of *Gabra1* transcription following TBI result in altered inhibitory synaptic neurotransmission in the hippocampus that may contribute to epileptogenesis. We hypothesize that after CCI inhibitory neurotransmission in the dentate gyrus will be altered in a fashion consistent with reduced $GABA_AR$ **α**1 subunit surface expression in association with JaK/STAT pathway activation and Gabra1 downregulation. To test this hypothesis, we will examine $GABA_AR$-mediated currents in dentate gyrus granule cells using whole-cell patch-clamp recordings in acute hippocampal slices from injured and uninjured mice.

3. Determine whether animals can be rescued from post-traumatic epilepsy development and/or progression via blockade of JaK/STAT pathway activation acutely after controlled cortical impact or chronically after onset of spontaneous seizures. We hypothesize that blockade of the JaK/STAT pathway will inhibit epilepsy development and/or progression after CCI. To address this hypothesis, we will inhibit STAT3 phosphorylation after CCI, either pharmacologically or using virally delivered short hairpin RNA (shRNAs) against JaK2 or STAT3, then use video electroencephalogram (EEG) monitoring to determine whether this treatment prevents or delays epilepsy development and/or progression.

Results of these studies will provide new information regarding the role of the JaK/STAT signaling cascade in regulation of brain inhibition and epileptogenesis after traumatic brain injury, and have the promise of leading to new therapies for the prevention or treatment of post-traumatic epilepsy.

BODY:

<u>Aim I: Performed in laboratory of Dr. Amy Brooks-Kayal at University of Colorado</u>

Task 1: Determine whether activation of the JaK/STAT pathway occurs following traumatic brain injury (TBI) and subsequent epileptogenesis. (Timeframe months 1-12)

1a. Induce TBI using the CCI model in adult CD-1 mice (200 mice- assuming that 25-30% loss due to death or suboptimal injury; Timeframe months 1-10)

Status: In progress
1. Established Controlled Cortical Impact (CCI) model as an experimental model of TBI at the University of Colorado, Denver UCD). This includes:

a. Purchase, installation and calibration of CCI apparatus

b. Testing of CCI apparatus to ensure reproducibility

c. Training of study personnel in surgical preparation for and performance of CCI injury. This included travel of 2 investigators (Dr. Frey and Dan Raible) to Dr. Smith's lab at the University of Kentucky for training in use of the CCI apparatus.

The CCI model was not in use at UCD prior to the start of this project and purchase, set-up and training was essential for the completion of all subsequent studies.

1b. Sacrifice mice at 6 and 24 hrs, 7 days and 10 weeks after CCI (150 mice; Timeframe months 1-4)
Status: In progress
1. Completed training of study personnel in non-traumatic dissection techniques to obtain cortical and hippocampal tissue samples from mouse brains
2. CCI induced in mice and animals sacrificed at 1, 3, 6, and 24 hour time points
Accomplishments:
Harvested tissue used for studies of STAT3 phosphorylation (see **1d** below)

1c. Measure levels of mRNA for JaK1 and 2, STAT1-5 in microdissected subregions of hippocampus (DG, CA1, CA3) and in cortex ipsilateral and contralateral to injury using quantitative reverse transcription polymerase chain reaction (qRT-PCR) (50 mice; Timeframe months 4-7).
Status: In progress
1. Completed training of study personnel in non-traumatic dissection techniques to obtain cortical and hippocampal tissue samples from mouse brains
2. Tested, validated and implemented mRNA protocols for measuring JaK1 and 2 and pSTAT1-5 using quantitative reverse transcription polymerase chain reaction (qRT-PCR)
Accomplishments: data collection in progress

1d. Measure protein levels of JaK1 and 2, pSTAT1-5 levels using western blotting in homogenates of microdissected brain regions (50 mice; Timeframe months 6-9).
Status: In progress
1. Completed training of study personnel in non-traumatic dissection techniques to obtain cortical and hippocampal tissue samples from mouse brains
2. Optimized and implemented Western blot protocols for protein measurements of JaK1 and 2 and pSTAT1-5 in mouse brain
Accomplishments:
Demonstrated that STAT3 phosphorylation is increased in injured mouse hippocampus after CCI. Hippocampus was dissected from injured and sham mice at 1, 3, 6, and 24 hours after CCI. Western blots of protein homogenates from whole hippocampus were probed with anti-pSTAT3 and STAT3 antibodies. Quantification of western blot analysis was performed and pSTAT3 levels were normalized to STAT3 levels and expressed as a % change compared to shams. These studies demonstrated that there was a significant increase in pSTAT3 levels at 6 hrs following CCI in injured hippocampus (* $P < 0.05$). *These results establish that there is an increase in activation of STAT3 following CCI in mice, similar in magnitude and time course to that seen in rat (see related studies,* ***Raible et al., J Neurotrauma, 2012 Sep 4. [Epub ahead of print], PMID: 22827467****. Appendix*

item #1), and support the feasibility of the proposed studies to examine the effect of JAK/STAT pathway blockade with WP1066 on subsequent development of post-traumatic epilepsy.
Supporting Data: See **Figure 1**

1e. Assess protein levels and regional/cellular expression of JaK1 and 2, pSTAT1-5 using fluorescent immunohistochemistry with co-staining for cell specific markers (50 mice; Timeframe months 9-12).
Status: Not yet initiated
Accomplishments: Not yet initiated
Supporting Data: Not yet initiated

Task 2: Determine whether upregulation of *ICER* transcription occurs following traumatic brain injury (TBI) and subsequent epileptogenesis. (Timeframe months 1-12: studies will overlap with Task 1 and be performed in same animals)
2a. Induce TBI using the CCI model in adult CD-1 mice (200 mice [same mice used in Task 1a]; months 1-10)
Status: In progress

2b. Sacrifice mice at 6 and 24 hrs, 7 days and 10 weeks after CCI (150 mice [same mice used in Task 1b]; months 1-4)
Status: In progress
1. Initiated experiments for 6 hour, 24 hour and 12 week time points after CCI in mice
Accomplishments: data collection in progress
Supporting Data: None

2c. Measure levels of mRNA for ICER in brain regions ipsilateral and contralateral to injury using qRT-PCR (50 mice [same 50 mice as used in Task 1c]; months 4-7).
Status: In progress
1. Completed training of study personnel in non-traumatic dissection techniques to obtain cortical and hippocampal tissue samples from mouse brains
2. Tested, validated and implemented mRNA protocols for measuring ICER using quantitative reverse transcription polymerase chain reaction (qRT-PCR) in RNA extracted from mouse brain
Accomplishments: data collection in progress
Supporting Data: None

2d. Measure protein levels of ICER using western blotting in brain regions (50 mice [same 50 mice as used in Task 1d]; Timeframe months 6-9).
Status: Not yet initiated
Accomplishments: Not yet initiated
Supporting Data: None

2e. Assess protein levels and regional/cellular expression of ICER using fluorescent immunohistochemistry (50 mice [same 50 mice as used in Task 1e]; Timeframe months 9-12).
Status: Not yet initiated
Accomplishments: Not yet initiated
Supporting Data: None

Task 3: Determine whether downregulation of *Gabra1* transcription occurs following traumatic brain injury (TBI) and subsequent epileptogenesis. (Timeframe months 1-12: studies will overlap with Tasks 1 and 2 and be performed in same animals)
3a. Induce TBI using the CCI model in adult CD-1 mice (200 mice- assuming that 25-30% loss due to death or suboptimal injury; months 1-10)
3b. Sacrifice mice at 6 and 24 hrs, 7 days and 10 weeks after CCI (150 mice [same used in Task 1b]; months 1-4)
Status: In progress
1. Established Controlled Cortical Impact (CCI) model as an experimental model of TBI at the University of Colorado, Denver as described above
2. Initiated experiments for 12 week time point
Accomplishments: tissue used to generate results reported in 3c and 3d below.
Supporting Data: none

3c. Measure levels of mRNA for *Gabr* subunits in brain regions ipsilateral and contralateral to injury using qRT-PCR (50 mice [same mice as used in Task 1c]; months 4-7).
Status: In progress
1. Completed training of study personnel in non-traumatic dissection techniques to obtain cortical and hippocampal tissue samples from mouse brains
2. Tested, validated and implemented mRNA protocols for measuring *Gabr* subunits using quantitative reverse transcription polymerase chain reaction (qRT-PCR) for mouse
Accomplishments: data collection in progress
Supporting Data: None

3d. Measure protein levels of *Gabr* subunit levels using western blotting in brain regions (50 mice [same mice used in Task 1d]; months 6-9).
Status: In progress
1. Established Controlled Cortical Impact (CCI) model as an experimental model of TBI at the University of Colorado, Denver as described above
2. Tested, validated and implemented protocols for measuring *Gabr* subunits in mouse
3. Initiated experiments for 12 week time point
Accomplishments:

1. Demonstrated that $GABA_A$ receptor subunit protein levels are not significantly changed in injured hippocampus 12 weeks after CCI in mouse. Western blots were performed using whole hippocampal protein collected from mice 12 week after CCI and probed with anti-$GABA_AR$ α1, α4, γ2, α2, α5 and β-actin antibodies. $GABA_AR$ subunit levels were normalized to β-actin levels and expressed as a percent change compared to Shams. N = 5 for CCI and N = 3 for Sham. Quantification of $GABA_AR$ subunit blots demonstrated no change in injured hippocampus relative to sham 12 weeks after CCI. Analysis of $GABA_AR$ subunits at additional timepoints (6 and 24 hrs, 7 days) is underway.

Supporting Data: see **Figure 2**

3e. Assess protein levels and regional/cellular expression of *Gabr* subunits using fluorescent immunohistochemistry (50 mice [same mice used in Task 1e]; months 9-12).
Status: Not yet initiated
Accomplishments: Not yet initiated

Aim 2: Performed in laboratory of Dr. Bret N. Smith at University of Kentucky

Task 1: Determine whether benzodiazepine modulation of IPSCs in dentate granule cells (DGCs) is altered after CCI and whether this alteration is prevented by inhibiting STAT3 phosphorylation with WP1066. (Timeframe months 1-18).

Task 1a. Induce TBI using CCI model in adult CD-1 mice (200 mice used, 20 sham-injured controls, 80 injured untreated, 20 sham-injured, WP1066-treated controls, 80 injured WP1066-treated; Timeframe months 1-18.

Status: In progress

1. Established precise parameters of Controlled Cortical Impact (CCI) model that result in epileptogenesis and development of epileptic phenotype after 8-12 weeks post-injury. This includes:
    a. Establish precise parameters of effective CCI to obtain epileptogenic phenotype. The problem to be overcome: obtaining epileptogenic phenotype with consistency is desirable in order to establish reliable baseline measurements from electrophysiological studies, but cortical damage after CCI is somewhat variable.
    b. Establish precise extent of injury with respect to distance from impact point, which is critical for determining the hippocampal regions affected by injury.
    c. Establish effect of on STAT3 phosphorylation.
    d. Establish effect of WP1066 on STAT3 phosphorylation after CCI.
    e. Establish treatment protocol effective in both laboratories. To ensure consistency of all outcomes across the labs at both University and Kentucky and University of Colorado, these experiments were done in concert and consultation with those performed by the other PIs. Initially, mice responded differently than expected to the WP1066 treatment, resulting in several failed experiments at both research sites. It was determined that details of drug storage and usage were likely culprits and drug ordering, preparation, and storage procedures were modified to address the issues. Once these technical issues were resolved, successful treatment and repeatability was established.

Accomplishments:

1. Determined precise parameters of effective CCI to obtain epileptogenic phenotype, including mossy fiber sprouting and hilar GABA neuron loss.
2. Determined that histopathological features (i.e., MFS and hilar GABA neuron loss) in the dentate gyrus consistent with epileptogenesis were limited to the areas immediately beneath the injury and extending 800 μm ventral to the injury. Contralaterally and at more ventral levels ipsilaterally, hippocampal pathology was not observed.
3. Determined that CCI increased STAT3 phosphorylation ipsilateral to the injury.
4. Determined that treatment at 30 and 90 min after CCI with WP1066 (50 mg/kg) inhibits STAT3 phosphorylation, but does not affect GABA neuron loss.

Supporting Data: see **Appendix item 2**, **Hunt et al., 2012,** Figure 2 for complete description of CCI parameters resulting in MFS and location of damage after injury. See **Figure 3 (below)** showing extent of hilar GABA neuron loss after injury in male mice

expressing GFP in a subset of hilar interneurons (GIN mice; FVB-Tg(GadGFP)4570Swn/J); see **Figure 4 (below)** showing increased STAT3 phosphorylation after CCI and inhibition of pSTAT3 by WP1066 treatment after CCI injury in our hands.

Task 1b. Measure effects of zolpidem on IPSCs in DGCs from WP1066-treated and untreated control mice and in mice shortly (i.e., 1-6 weeks) after CCI injury). (100 mice needed, 10 sham-injured controls, 40 injured untreated, 10 sham-injured, WP1066-treated controls, 40 injured WP1066-treated; subset of mice in Task 1a; Timeframe months 1-9).

Status: not yet initiated
Accomplishments: none.
Supporting Data: none

Task 1c. Measure effects of zolpidem on IPSCs in DGCs from WP1066-treated and untreated control mice and in mice 6-10 weeks after CCI injury. (100 mice needed, 10 sham-injured controls, 40 injured untreated, 10 sham-injured, WP1066-treated controls, 40 injured WP1066-treated; subset of mice in Task 1a; Timeframe months 4-18)

Status: In progress

1. Establishing precise IPSC parameters in control and CCI-injured mice at 6-10 weeks after injury. This includes:
   a. Train personnel in recording and analysis techniques.
   b. Obtain sufficient numbers of recordings to sufficiently identify differences
2. Effects of zolpidem on IPSCs in four treatment groups.
   a. Obtain license to purchase benzodiazepine agonists
   b. Use zolpidem in recordings from DGCs in slices from four treatment groups

Accomplishments:

1. Personnel (Graduate student Corwin Butler) fully trained to perform recordings;
2. DEA license application procedure in process.

Supporting Data: none.

Task 1d. Perform Timm histological analysis, to detect mossy fiber sprouting in all slices from which recordings are made. (200 mice needed; same mice as in Tasks 1a-c; Timeframe months 1-18).

Status: In progress
Accomplishments:

1. Timm staining parameters for sham control and CCI-injured established;
2. Timm staining for WP1066-treated are in process.

Supporting Data: See **Appendix item 2, Hunt et al., 2012**, figures 1 and 2.

Task 2: Determine if furosemide modulation of IPSCs in DGCs is altered after CCI and if inhibiting STAT3 phosphorylation with WP1066 prevents the alteration. (Timeframe: months 19-36)

Task 2a. Induce TBI using CCI model in adult CD-1 mice (200 mice used, 20 sham-injured controls, 80 injured untreated, 20 sham-injured, WP1066-treated controls, 80 injured WP1066-treated; Timeframe months 19-36).

Status: in progress
Accomplishments: Accomplishments identical to Task 1, 1a.
Supporting Data: Same as Task 1, 1a.

Task 2b. Measure effects of furosemide on IPSCs in DGCs from WP1066-treated and untreated control mice and in mice 1-6 weeks after CCI injury. (100 mice needed, 10 sham-injured controls, 40 injured untreated, 10 sham-injured, WP1066-treated controls, 40 injured WP1066-treated; subset of mice in Task 2a; Timeframe months 19-27).

Status: not yet initiated
Accomplishments: none.
Supporting Data: none

Task 2c. Measure effects of furosemide on IPSCs in DGCs from WP1066-treated and untreated control mice and in mice 6-10 weeks after CCI injury (months 4-18). 100 mice needed, 10 sham-injured controls, 40 injured untreated, 10 sham-injured, WP1066-treated controls, 40 injured WP1066-treated; subset of mice in Task 2a; Timeframe months 19-36).

Status: not yet initiated
Accomplishments: none.
Supporting Data: none

Task 2d. Perform Timm histological analysis, to detect mossy fiber sprouting in all slices from which recordings are made. (200 mice needed; same mice as in Tasks 2a-c; Timeframe months 19-36).

Status: in progress
Accomplishments: Accomplishments identical to Task 1, 1d.
Supporting Data: Same as Task 1, 1d.

Task 3: Determine if THIP-induced tonic GABA currents in DGCs are altered after CCI and if the alteration is prevented by inhibiting STAT3 phosphorylation with WP1066. (Timeframe months 10-27)

Task 3a. Induce TBI using CCI model in adult CD-1 mice (200 mice used, 20 sham-injured controls, 80 injured untreated, 20 sham-injured, WP1066-treated controls, 80 injured WP1066-treated; Timeframe months 10-27)

Status: in progress
Accomplishments: Accomplishments identical to Task 1, 1a.
Supporting Data: Same as Task 1, 1a.

Task 3b. Measure THIP-induced tonic GABA current in DGCs from WP1066-treated and untreated control mice and in mice 1-6 weeks after CCI injury. (100

mice needed, 10 sham-injured controls, 40 injured untreated, 10 sham-injured, WP1066-treated controls, 40 injured WP1066-treated; subset of mice in Task 3a; Timeframe months 10-18).

Status: not initiated

Accomplishments: none

Supporting Data: Same as Task 1, 1a.

Task 3c. Measure THIP-induced tonic GABA current in DGCs from WP1066-treated and untreated control mice and in mice 6-10 weeks after CCI injury (months 4-18). 100 mice needed, 10 sham-injured controls, 40 injured untreated, 10 sham-injured, WP1066-treated controls, 40 injured WP1066-treated; subset of mice in Task 3a; Timeframe months 19-27).

Status: in progress

Accomplishments:

1. Trained Graduate student Corwin Butler to perform all electrophysiological experiments and analyses.
2. Determined that THIP-induced tonic GABA current in DGCs 6-10 weeks post-injury are reduced in amplitude relative to controls and contralateral DGCs. Based on preliminary results from collaborators in Colorado and on data published recently elsewhere, these experiments were initiated to identify potential functional changes due to altered $\alpha 4/\delta$ subunit-containing GABA receptor expression weeks after injury, corresponding to time points where epilepsy is established in this model.
3. WP1066-treated control and CCI-injured animals have been prepared

Supporting Data: see **Figure 5** for demonstration of THIP-induced changes in tonic GABA current after 6-10 weeks post injury.

Task 3d. Perform Timm histological analysis, to detect mossy fiber sprouting in all slices from which recordings are made. (200 mice needed; same mice as in Tasks 3a-c; Timeframe months 10-27).

Status: in progress

Accomplishments: Accomplishments identical to Task 1, 1d.

Supporting Data: Same as Task 1, 1d.

Aim 3: Performed in laboratory of Dr. Amy Brooks-Kayal at University of Colorado

Task 1: Determine whether inhibition of STAT3 phosphorylation after CCI using WP1066 inhibits post-traumatic epilepsy development (PTE) (Timeframe months 12-21)

1a. Induce TBI using the CCI model in adult CD-1 mice and administer WP1066 or vehicle immediately after injury (200 mice- assuming that 30% loss due to death, suboptimal injury, or loss of implanted recording electrodes; months 12-18)

1b. Sacrifice mice at 6 hrs, 24 hrs and 7 days after CCI to assess molecular and histochemical effects of WP1066 (120 mice [assuming that 25-30% loss due to death, suboptimal injury]; months 12-15)

Status: In progress

1. Established Controlled Cortical Impact (CCI) model as an experimental model of TBI at the University of Colorado, Denver as detailed above
2. Established protocol for WP1066 or vehicle administration

Accomplishments:

1. We performed multiple experiments to identify a dosing paradigm for WP1066 that resulted in inhibition of STAT3 phosphorylation following CCI in mouse.
2. First we administered WP1066 50 mg/kg ip either 15 min before or 5 min after CCI injury and harvested hippcampal tissue at 6 hrs after CCI. Western blots of protein homogenates from whole hippocampus were then probed with anti-pSTAT3 and STAT3 antibodies. Quantification of western blot analysis was performed and pSTAT3 levels were normalized to STAT3 levels and expressed as a % change compared to shams. These studies demonstrated that there was no significant difference in pSTAT3 levels at 6 hrs following CCI in injured hippocampus between vehicle treated and WP1066 treated groups (see **Figure 6**)
3. We next examined if 15 minute pre-treatment with a WP1066 analogue with enhanced stability (WP117) at varying doses would inhibit phosphorylation of STAT3 in injured hippocampus 1 hour after CCI. Western blots of protein homogenates from whole hippocampus were performed as described above. These studies demonstrated that there was no significant difference in pSTAT3 levels at 1 hr following CCI in injured hippocampus between vehicle treated and WP117 treated groups, and suggested that very early treatment with WP1066 or analogue did not inhibit STAT3 phosphorylation (see **Figure 7**).
4. Finally we administered WP1066 50 mg/kg ip 30 and 90 minute post-CCI and harvested tissue at multiple timepoints after CCI. These studies demonstrated that WP1066 administered at 30 and 90 minutes after CCI inhibits STAT3 phosphorylation in injured hippocampus 3, 6 and 24 hours after CCI (see **Fig. 8**). This treatment protocol has thus now been shown to be successful in both the Smith and Brooks-Kayal labs

Supporting Data: **Figures 6, 7, 8**

1c. Measure levels of mRNA for JaK/STAT, ICER and *Gabr* subunits in brain regions ipsilateral and contralateral to injury using qRT-PCR (30 mice [subset of mice as in Task 1b]; months 12-15).

Status: In progress

1. Completed training of study personnel in non-traumatic dissection techniques to obtain cortical and hippocampal tissue samples from mouse brains
2. Tested, validated and implemented mRNA protocols for measuring JaK/STAT, ICER and *Gabr* subunits using quantitative rtPCR in mouse

Accomplishments: data collection in progress

Supporting Data: None

1d. Measure protein levels of JaK/STAT, ICER and *Gabr* subunits using western blotting in brain regions (30 mice [subset of mice as used in Task 1b]; months 15-18).

Status: In progress for pSTAT3

1. Completed training of study personnel in non-traumatic dissection techniques to obtain cortical and hippocampal tissue samples from mouse brains
2. Tested, validated and implemented protocols for measuring JaK/STAT, ICER and *Gabr* subunit levels using western blotting in mouse

Accomplishments:

1. Demonstrated that 30 and 90 minute post-treatment with WP1066 inhibits phosphorylation of STAT3 in injured hippocampus 3, 6 and 24 hours after CCI.

Supporting Data: see **Figure 8**

1e. Assess protein levels and regional/cellular expression of JaK/STAT, ICER and *Gabr* subunits using fluorescent immunohistochemistry (30 mice [subset of mice used in Task 1b]; months 18-21).
Status: Not yet initiated
Accomplishments: Not yet initiated. Supporting Data: None

1f. Assess cell injury and neurogenesis using Fluoro-Jade, TUNEL, caspase and nestin staining; (30 mice [same mice used in Task 1e] months 18-21).
Status: Not yet initiated
Accomplishments: Not yet initiated. Supporting Data: None

1g. Assess glial proliferation using GFAP and ALDH1L1 staining (30 mice [same mice used in Task 1e]; months 18-21).
Status: Not yet initiated
Accomplishments: Not yet initiated. Supporting Data: None

1h. Implant and video-EEG monitor a subset of mice from 6-10 weeks post injury (80 mice - assuming 30% loss due to death with surgery or loss of implanted recording electrodes; months 16-20)
Status: In progress
1. Established collaborative relationship with University of Colorado Small Animal Neurophysiology core facility, an electrically clean facility on campus for long-term rodent video-EEG monitoring
3. Completed training of study personnel in EEG electrode implantation
4. Optimized EEG electrode implantation protocol for CCI animals. Because the skull and the underlying brain is disrupted during CCI, these animals require a specialized implantation protocol to minimize the impact of the skull and brain disruption on the quality of the collected signal (see **Figure 9**).
5. Implanted 3 animals with CCI and 3 sham animals at 6 weeks after injury.
6. EEGs were recorded and interpreted for these animals from 7-12 weeks after injury.
Accomplishments:
1. Demonstrated that mice subjected to CCI had bursts of spikes on their EEG, indicative of cortical hyperirritability, potentially injury-related. None of the sham-injured animals had interictal similar findings. No seizures were recorded. These studies demonstrate that mice have a propensity for seizures after CCI but may need to be recorded later than 12 weeks post CCI to document spontaneous seizures. We plan to increase recording duration in future studies and may try adjusting the CCI parameters to maximize seizure occurrence.
Supporting Data: **Figure 9 and 10**

1i. Assess mossy fiber sprouting, cell loss and glial proliferation 10 weeks post injury using Timm and Nissl staining (20 mice [subset of mice used in Task 1h]; months 20-21).
Status: Not yet initiated
Accomplishments: Not yet initiated. Supporting Data: None

1j. Assess pSTAT3, ICER and *Gabra1* protein levels 10 weeks post injury (20 mice [subset of mice used in Task 1h]; months 20-21).
Status: In progress
Accomplishments:
1. Completed training of study personnel in dissection techniques as above
2. Tested, validated and implemented protocols for measuring JaK/STAT, ICER and *Gabr* subunit levels using western blotting in mouse as above
3. Initiated experiments for 12 week time points
Supporting Data: See **Figure 2**.

1k. Assess ICER and *Gabra1* mRNA levels 10 weeks post injury (20 mice [subset of mice used in Task 1h]; months 20-21).
Status: Not yet initiated
Accomplishments: Not yet initiated. Supporting Data: None

Task 2: Determine whether inhibition of STAT3 phosphorylation after CCI using shRNAs inhibits PTE development (Timeframe months 21-29)
2a. Induce TBI using the CCI model in adult CD-1 mice 1 week after administration of shRNAs for JaK2 or STAT3 or scrambled shRNAs (400 mice- assuming that 30% loss due to death, suboptimal injury, or loss of implanted recording electrodes; months 21-26)
2b. Sacrifice mice at 6 hrs, 24 hrs and 7 days after CCI to assess molecular and histochemical effects of shRNAs (240 mice [assuming that 25-30% loss due to death, suboptimal injury]; months 21-24)
2c. Measure levels of mRNA for JaK/STAT, ICER and *Gabr* subunits in brain regions ipsilateral and contralateral to injury using qRT-PCR (60 mice [subset of mice as in Task 2b]; months 21-24).
2d. Measure protein levels of JaK/STAT, ICER and *Gabr* subunit levels using western blotting in brain regions (60 mice [subset of mice as used in Task 2b]; months 22-25).
2e. Assess protein levels and regional/cellular expression of JaK/STAT, ICER and *Gabr* subunits using fluorescent immunohistochemistry (60 mice [subset of mice used in Task 2b]; months 24-27).
2f. Assess cell injury and neurogenesis using Fluoro-Jade, TUNEL, caspase and nestin staining; (60 mice [same mice used in Task 2e] months 24-27).
2g. Assess glial proliferation using GFAP and ALDH1L1 staining (60 mice [same mice used in Task 2e]; months 24-27).
2h. Implant and video-EEG monitor a subset of mice from 6-10 weeks post injury (160 mice - assuming 30% loss due to death with surgery or loss of implanted recording electrodes; months 22-28)
2i. Assess mossy fiber sprouting, cell loss and glial proliferation 10 weeks post injury using Timm and Nissl staining (40 mice [subset of mice used in Task 2h]; months 24-26).
2j. Assess pSTAT3, ICER and *Gabra1* protein levels 10 weeks post injury (40 mice [subset of mice used in Task 2h]; months 25-28).
2k. Assess ICER and *Gabra1* mRNA levels 10 weeks post injury (40 mice [subset of mice used in Task 2h]; months 27-29).
Status: Not yet initiated. Accomplishments: None. Supporting Data: None

Task 3: Determine whether inhibition of STAT3 phosphorylation with WP1066 in animals with PTE reduces seizure frequency and/or inhibits PTE progression (Timeframe months 30-36)

3a. Induce TBI using the CCI model in adult CD-1 mice (50 mice- assuming 25-30% loss due to death or suboptimal injury; months 30-31)

3b. Implant with subdural electrodes and video-EEG monitor mice from 6-8 weeks post injury as baseline (40 mice [assuming 30% loss due to death with surgery or loss of implanted recording electrodes]; months 32-34)

3c. Administer WP1066 50-100 mg/kg or vehicle daily for 2 weeks and continue video-EEG monitoring (30 mice; months 32-35)

Status: In progress

Accomplishments:

1. Established Controlled Cortical Impact (CCI) model as described above
2. Established protocol for WP1066 or vehicle administration as described above
3. Established collaborative relationship with University of Colorado Small Animal Neurophysiology core facility as described above
4. Completed training of study personnel in EEG electrode implantation
5. Optimized EEG electrode implantation protocol for CCI animals as above
6. Implanted 3 animals with CCI, 3 sham animals at 6 weeks after injury.
7. EEGs recorded and interpreted for these animals from 7-12 weeks after injury. All animals with CCI had bursts of spikes on their EEG, indicative of injury-related cortical hyperirritability, as described above

Supporting Data: **Figures 9 and 10**

3d. Sacrifice animals and assess protein and mRNA levels for Gabr subunits and perform histological assessment of cell counts and mossy fiber sprouting (30 mice [same mice used in 3c]; months 34-36)

Status: In progress

1. Completed training of study personnel in non-traumatic dissection as above
2. Tested, validated and implemented protocols for measuring JaK/STAT, ICER and *Gabr* subunit levels using western blotting and RT-qPCR in mouse brain
3. Initiated experiments for 12 week time points

Accomplishments: Data collection in progress

Supporting Data: See **Figure 2**

KEY RESEARCH ACCOMPLISHMENTS:

- We demonstrated that STAT3 is phosphorylated in injured mouse hippocampus 1, 3, 6, and 24 hours after CCI. These studies confirm that the magnitude and timecourse of pSTAT3 increases are similar in mouse CCI as we have recently reported in rat FPI model and establish feasibility of proposed studies to examine the inhibition of pSTAT3 on epilepsy development after CCI in mouse.
- We determined that THIP-induced tonic GABA current in DGCs 6-10 weeks post-CCI injury are reduced in amplitude relative to controls and contralateral DGCs. These results suggest that there may be an alteration in $\alpha 4/\delta$ subunit-containing GABA receptor expression weeks after injury, corresponding to time points where epilepsy is established in this model.
- We demonstrated that treatment with WP1066 50 mg/kg ip at 30 and 90 minute post-CCI inhibits phosphorylation of STAT3 in injured hippocampus 3, 6 and 24 hours

after CCI. This establishes feasibility of using WP1066 to examine the effects of inhibition of pSTAT3 on epilepsy development after CCI in mouse.
- We demonstrated that all mice that were EEG-monitored following CCI had bursts of spikes on their EEG, indicative of injury related cortical hyperirritability consistent with a high potential to develop epilepsy.

RELATED RESEARCH ACCOMPLISHMENTS (not funded by DOD grant)

- In the rat, quantification of $GABA_AR$ subunit α1 blots showed a significant decrease at 24 hours through 1 week after FPI injury relative to controls. Quantification of $GABA_AR$ subunit α4 blots showed a significant increase at 24 hours after FPI but a significant decrease relative to controls at one week after injury.
- In the rat, $GABA_AR$ α1 subunit mRNA levels are decreased and $GABA_AR$ α4 subunit mRNA levels are increased in injured hippocampus 6 hours after FPI.
- In the rat, $GABA_AR$ subunit γ2 is decreased while α2 and α5 are unchanged 1 week after FPI. In mice, $GABA_AR$ subunit levels are not significantly changed 12 weeks after CCI. Data for earlier time points in mouse are pending.
- In the rat, phosphorylated STAT3 levels were increased in injured hippocampus as early as 6 hours after FPI. By one week, there was no appreciable activation.
- In the rat, ICER and Egr3 expression are increased in injured hippocampus 6 hours after FPI.

REPORTABLE OUTCOMES: manuscripts, abstracts, presentations

1. Hunt, R.F., Haselhorst, L.A., Schoch, K.M., Bach, E.C., Rios-Pilier, J., Scheff, S.W., Saatman, K.E., and Smith, B.N. (2012) Posttraumatic mossy fiber sprouting is related to the degree of cortical damage in three mouse strains. *Epilepsy Res.* 99:167-170. This paper reported refinement of the CCI model in mice and establishment of injury parameters
2. Butler, CR, Boychuk, JA, Raible, D, Frey, L, Brooks-Kayal, A.R., Smith, BN (2012). JAK/STAT Activation and GABA Neuron Loss After Focal Traumatic Brain Injury in Mice, Society for Neuroscience abstract and Boychuk JA, Butler CR, Raible D, Frey L, Brooks-Kayal AR and Smith BN (2012), Focal traumatic brain damage results in localized GABA neuron loss and JAK/STAT activation early following injury, American Epilepsy Society abstract. These abstracts report that pSTAT3 was increased ipsilateral to a CCI injury in mice and that administration of the STAT3 inhibitor WP1066 in mice entered the brain and inhibited pSTAT3 production. GABA neuron loss was identified ipsilateral to the injury, but the loss was not prevented by the inhibitor.

RELATED PUBLICATIONS (not funded by DOD grant):

1. Raible DJ, Frey LC, Cruz Del Angel Y, Russek SJ, and Brooks-Kayal AR (2012) $GABA_A$ Receptor Regulation after Experimental Traumatic Brain Injury. *J Neurotrauma*, [Epub ahead of print], PMID: 22827467 (Appendix item #1). This paper reported that TBI in rats (FPI) resulted in transcriptional changes in GABA(A) receptor subunit expression following FPI including $GABA_AR$ α1 and

α4 subunits, and an associated increase in the phosphorylation of STAT3 and an increase in the expression of Egr3 and ICER.

Conclusions

In the first year of DOD CDMRP funding, the CCI model has been successfully used in mice at both University of Colorado and University of Kentucky, making future experiments feasible. Essential baseline data has been obtained on optimal techniques for molecular, anatomical, electrophysiological and neurophysiological (EEG) analysis in the mouse CCI model, and on GABA(A) receptor, JAK/STAT pathway and cell death alterations following TBI. Specific outcome measures to assess the rigor of the model in individual experiments for all molecular, electrophysiological and other studies have been established. Training has been completed for all personnel on all necessary techniques. Parameters of controls for several experiments have been established. Moreover, issues with the JAK/STAT3 inhibitor (WP1066) have been resolved and the drug is now successfully used in all labs. We established that EEG in mice become hyperexcitable by 12 weeks after CCI but do not yet demonstrate electrographic seizures. Going forward we plan to extend our EEG recording time and attempt some adjustments to CCI parameters in order to maximize identification of spontaneous seizures in this model.

Appendices

1. Raible DJ, Frey, LC, Cruz Del Angel Y, Russek SJ, and Brooks-Kayal AR (2012) $GABA_A$ Receptor Regulation after Experimental Traumatic Brain Injury. *J Neurotrauma*, [Epub ahead of print], PMID: 22827467
2. Hunt, R.F., Haselhorst, L.A., Schoch, K.M., Bach, E.C., Rios-Pilier, J., Scheff, S.W., Saatman, K.E., and Smith, B.N. (2012) Posttraumatic mossy fiber sprouting is related to the degree of cortical damage in three mouse strains. *Epilepsy Res.* 99:167-170.
3. Butler, CR, Boychuk, JA, Raible, D, Frey, L, Brooks-Kayal, A.R., Smith, BN (2012). JAK/STAT Activation and GABA Neuron Loss After Focal Traumatic Brain Injury in Mice. Society for Neuroscience abstract.
4. Boychuk JA, Butler CR, Raible D, Frey L, Brooks-Kayal AR and Smith BN (2012). Focal traumatic brain damage results in localized GABA neuron loss and JAK/STAT activation early following injury. American Epilepsy Society abstract.

SUPPORTING DATA:

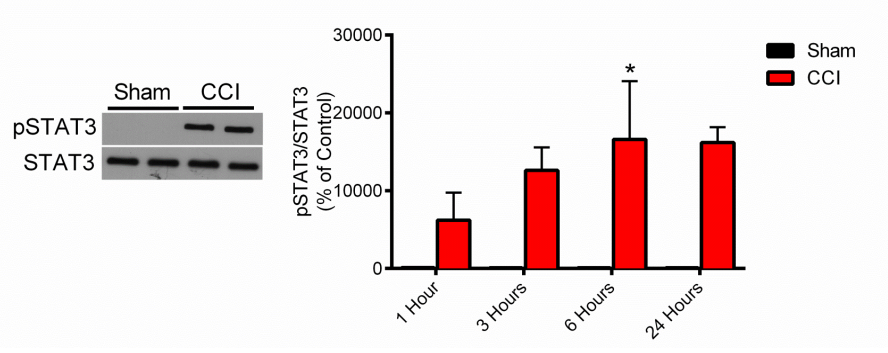

Figure 1: STAT3 is phosphorylated in injured hippocampus 1, 3, 6, and 24 hours after CCI. (A) Representative western blots of protein homogenates from whole hippocampus of mice 6 hours after CCI probed with anti-pSTAT3 and STAT3 antibodies. (B) Quantification of western blot analysis of pSTAT3 levels from CCI and sham mice. pSTAT3 levels were normalized to STAT3 levels and expressed as a % change compared to shams. * $P < 0.05$.

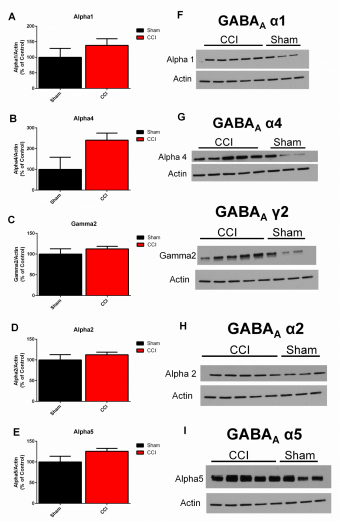

Figure 2: GABA$_A$ receptor subunits are not significantly changed 12 weeks after CCI. (A-E) Quantification of GABA$_A$R subunit blots 12 weeks after CCI relative to sham. (F-I) Representative western blots from whole hippocampal protein collected from mice 12 week after CCI probed with anti-GABA$_A$R α1, α4, γ2, α2, α5 and β-actin antibodies. GABA$_A$R subunit levels were normalized to β-actin levels and expressed as a percent change compared to Shams. N = 5 for CCI and N = 3 for Sham.

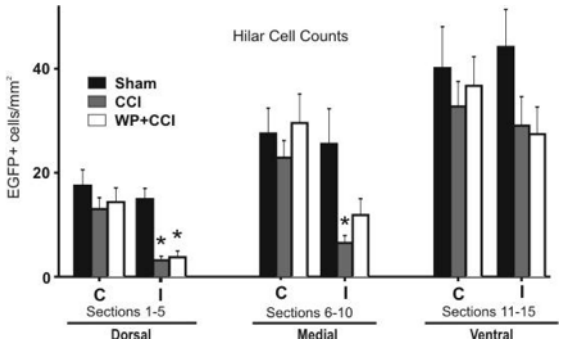

Figure 3: Hilar interneuron counts in Sham, CCI, and CCI with WP1066 treatment. Cell density was reduced in dorsal, but not ventral, hippocampus after CCI. WP1066 treatment did not prevent the cell loss after CCI (ANOVA, Tukey's post hoc; $p<0.05$).

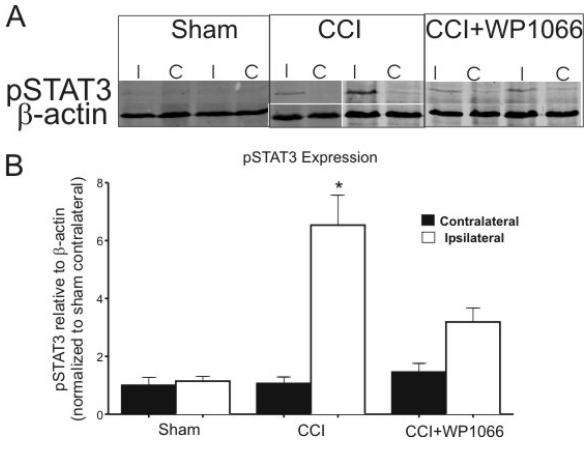

Figure 4: Western blot and representative bar graph of pSTAT3 expression relative to β-actin content in CCI, Sham, and CCI with WP1066 treatment. Expression of pSTAT3 is significantly increased in the ipsilateral hemisphere of CCI hippocampus compared to Sham 24 hr after injury. Expression of pSTAT3 was inhibited after WP1066 treatment relative to CCI ($P<0.05$; ANOVA, Tukey's post hoc), and was similar to levels seen in Shams ($P>0.05$; ANOVA, Tukey's post hoc).

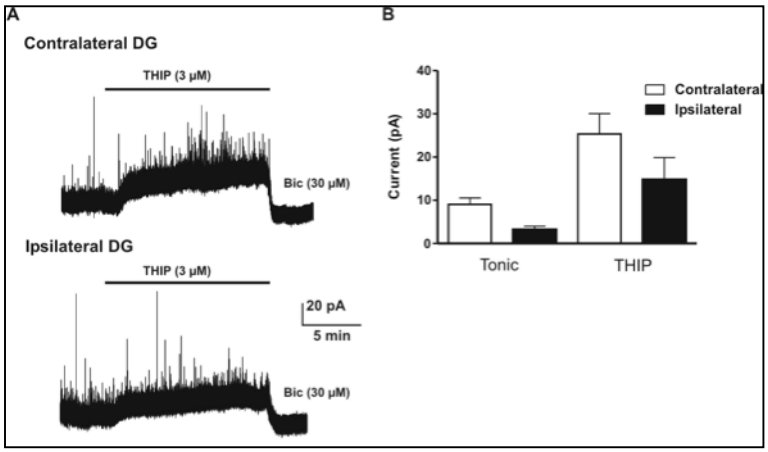

Figure 5. THIP-activated, tonic GABA currents are reduced in dentate granule cells after CCI. A. Representative traces of DGCs located contralateral (upper left) or ipsilateral (lower left) to head injury with CCI. DGCs were voltage clamped at 0 mV (close to the reversal of glutamatergic currents) and recorded in three phases: baseline, THIP (3 μM) and Bicuculline (Bic; 30 μM). **B**. Group data of tonic GABA current and THIP responses of DGCs located either contralateral or ipsilateral to CCI.

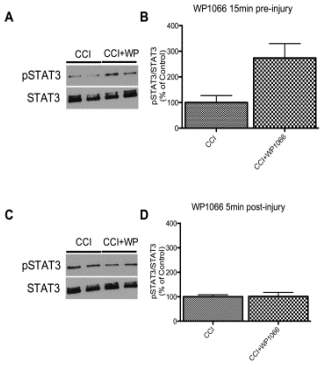

Figure 6: IP administration of WP1066 at 15 min before or 5 minutes after CCI did not inhibit phosphorylation of STAT3 in injured hippocampus 6 hours after CCI. (A,C) Representative western blots of protein homogenates from ipsilateral whole hippocampus (relative to CCI) of mice treated with WP1066 or DMSO (15 minutes prior to injury for A) or (5 minutes after injury for B) and sacrificed 6 hours after CCI probed with pSTAT3 and STAT3 antibodies. (B,D) Quantification of pSTAT3 levels from CCI + 50mg/Kg of WP1066 and CCI + DMSO mice. pSTAT3 levels were normalized to STAT3 levels and expressed as a percent change compared to sham controls. (15 minutes pre-treatment n = 2 for CCI, 3 for CCI+wp1066; 5 minute post-treatment n = 2 for CCI, 3 for CCI+wp1066).

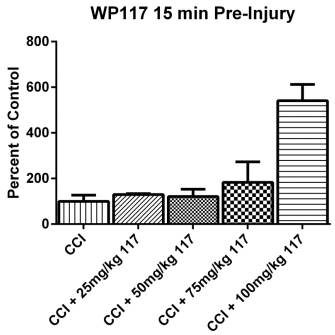

Figure 7: 15 minute pre-treatment IP administration of WP1066 analog (WP117) at varying does did not inhibit phosphorylation of STAT3 in injured hippocampus 1 hour after CCI. Quantification of pSTAT3 levels from CCI + 25mg/kg WP117, CCI + 50mg/kg WP117, CCI + 75mg/kg WP117, CCI + 100 mg/kg WP117 and CCI + DMSO mice. n = 6 for CCI, n = 4 for CCI + 25mg/kg WP117; n = 7 for CCI + 50mg/kg WP117; n = 2 for CCI + 75mg/kg WP117; n = 3 for CCI + 100mg/kg WP117.

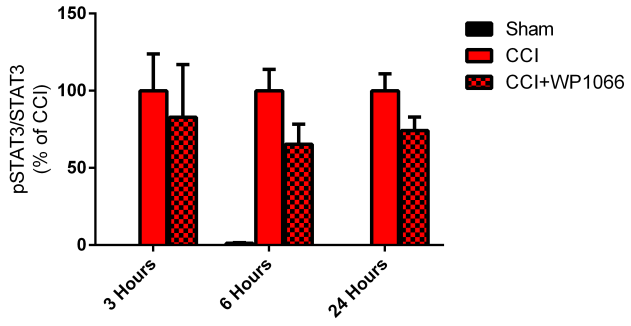

Figure 8: 30 and 90 minute post-treatment of WP1066 does appear to be inhibiting phosphorylation of STAT3 in injured hippocampus 3, 6 and 24 hours after CCI. Quantification of pSTAT3 levels from CCI + 50mg/kg of WP1066 30 and 90 minutes post-treatment, CCI + DMSO, or Sham injured animals sacrificed 3, 6, 24 hours after injury. n = 3 for CCI and n = 4 for CCI + WP1066 at 3 hours; n = 5 for Sham, n = 7 for CCI and n = 4 for CCI + WP1066 at 6 hours; n = 2 for CCI and n = 2 for CCI + WP1066 at 24 hours

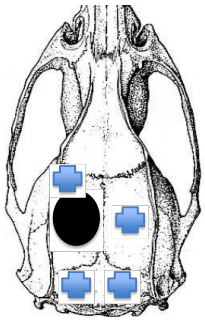

Figure 9: Representative image of the mouse skull with electrode implants and craniotomy. The black circle represents the area of skull which was removed so that the CCI injury could be implemented. The crosses represent the placement of the electrodes (2 recording, 1 ground and reference).

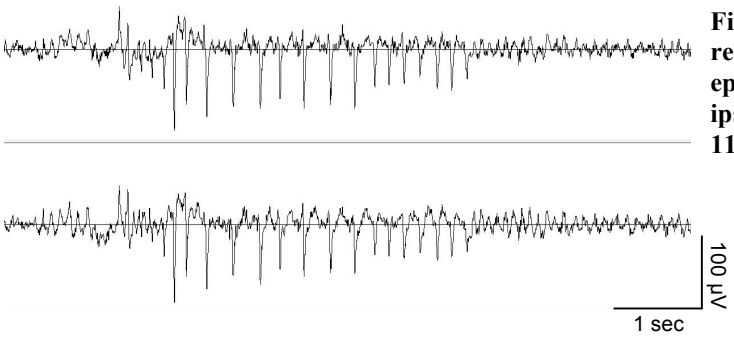

Figure 10: Representative EEG recording of a spontaneous epileptiform burst in both the ipsilateral and contralateral leads 11 weeks after CCI.

Focal traumatic brain damage results in localized GABA neuron loss and JAK/STAT activation early following injury

JA Boychuk[1], CR Butler[1], D Raible[2], L. Frey[3], AR Brooks-Kayal[4,5] and BN Smith[1,6]

[1]Department of Physiology, College of Medicine, University of Kentucky, KY 40536
[2]Neuroscience Graduate Program, University of Colorado Denver, Aurora CO 80045
[3]Department of Neurology, University of Colorado Denver, Aurora CO 80045
[4]Department of Pediatrics, Neurology, and Pharmaceutical Sciences, University of Colorado Denver, Aurora CO 80045
[5]The Childrens Hospital Colorado, Aurora, CO 80045
[6]Spinal Cord and Brain Injury Research Center (SCoBIRC), University of Kentucky

Rationale

Traumatic brain injury (TBI) is a leading cause of acquired temporal lobe epilepsy (TLE), yet the mechanisms underlying posttraumatic epileptogenesis are not known. TBI is associated with cell loss and changes in cellular and synaptic signaling in cortical structures. Activation of the JAK/STAT3 pathway has been implicated as a participant in the reactive plasticity associated with epileptogenesis. Here, a murine model of TBI that results in spontaneous seizures was used to examine aspects of hippocampal GABA network modification shortly after brain injury. We tested the hypothesis that regional JAK/STAT3 pathway modulation and hilar GABAergic interneuron loss occurs shortly after focal brain injury.

Methods

Severe controlled cortical impact (CCI; impact depth= 1.0 mm) was administered to male mice at 6-8 weeks of age. 24 hours post- injury, hippocampi from male CD-1 mice were isolated and processed for western blot analysis of STAT3 and pSTAT3 proteins, with the latter used as a marker of JAK/STAT3 activation. The effectiveness of the STAT3 inhibitor WP1066 (EMD Millipore; 30 and 90 min post-CCI; 50mg/kg; i.p.) on blocking JAK/STAT3 activation was also tested. Hilar GABA cell loss 2-3 days following CCI was examined in male mice expressing GFP in a subset of hilar interneurons (GIN mice; FVB-Tg(GadGFP)4570Swn/J). Coronal sections (30 μm) were sampled in a 1 in 5 series. GFP-positive cell counts and hilus area were analyzed using a laser scanning confocal microscope (Zeiss, LSM 5 LIVE).

Results

At 24 hours following CCI an increase in hippocampal pSTAT3 protein expression ipsilateral to the injury was observed, relative to either the contralateral hemisphere or Sham-operated controls. Both Sham-operated controls and the contralateral hemisphere of injured animals exhibited low levels of hippocampal pSTAT3 protein expression. Administration of the STAT3 inhibitor WP1066 inhibited pSTAT3 protein expression in the hippocampus ipsilateral to the injury, but had little effect on the contralateral hemisphere of injured animals or Sham operated controls. Quantification of GFP positive cells from GIN mice revealed a decrease in the number of hilar interneurons within dorsal hippocampus ipsilateral to the injury relative to the contralateral hemisphere or to Sham-

operated controls. Preliminary data indicate that administration of WP1066 did not inhibit hilar GABA cell loss ipsilateral to the injury.

Conclusions

Early time-points following brain injury with CCI are associated with changes in hippocampal GABA networks. These changes are prominent in the hippocampus ipsilateral to the injury and include a loss of hilar GABAergic interneurons as well as activation of the JAK/STAT signaling pathway. Previous studies have associated both of these changes with alterations in GABA signaling associated with TLE. Alterations in hippocampal GABA neuron function following head injury may support the eventual expression of spontaneous seizures in posttraumatic epilepsy.

Character limit: 3200 characters from title, abstract and spaces not including authors, institutions, tables or images.

Characters (-authors/affiliations) = 3123

Epilepsy Research (2012) **99**, 167—170

journal homepage: www.elsevier.com/locate/epilepsyres

SHORT COMMUNICATION

Posttraumatic mossy fiber sprouting is related to the degree of cortical damage in three mouse strains

Robert F. Hunt[a], Laura A. Haselhorst[a], Kathleen M. Schoch[a,d], Eva C. Bach[a], Jennifer Rios-Pilier[a], Stephen W. Scheff[b,c,d], Kathryn E. Saatman[a,d], Bret N. Smith[a,d,*]

[a] *Department of Physiology, University of Kentucky, Lexington, KY 40536-0298, USA*
[b] *Department of Anatomy and Neurobiology, University of Kentucky, Lexington, KY 40536-0298, USA*
[c] *Sanders-Brown Center on Aging, University of Kentucky, Lexington, KY 40536-0298, USA*
[d] *Spinal Cord and Brain Injury Research Center, University of Kentucky, Lexington, KY 40536-0298, USA*

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KEYWORDS
Contusion;
Dentate gyrus;
Epileptogenesis;
Epilepsy model;
Seizure;
Traumatic brain injury

Summary Controlled cortical impact injury was used to examine relationships between focal posttraumatic cortical damage and mossy fiber sprouting (MFS) in the dentate gyrus in three mouse strains. Posttraumatic MFS was more robust when cortical injury impinged upon the hippocampus, versus contusions restricted to neocortex, and was qualitatively similar among CD-1, C57BL/6, and FVB/N background strains. Impact parameters influencing injury severity may be critical in reproducing epilepsy-related changes in neurotrauma models.
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Introduction

Mossy fiber sprouting (MFS) into the inner molecular layer of the dentate gyrus is a consistent marker of the epileptic dentate gyrus after traumatic brain injury (TBI) in humans (Swartz et al., 2006) and animals (Kharatishvili et al., 2006; Hunt et al., 2009, 2010, 2011). MFS is generally more robust after severe versus mild TBI (Santhakumar et al., 2000; Kharatishvili et al., 2006, 2007; Hunt et al., 2009), but responses in posttraumatic animals can be highly variable. We recently described the development of posttraumatic epilepsy (PTE) and localized, robust MFS and synaptic reorganization 6—12 weeks after controlled cortical impact (CCI) injury in mice (Hunt et al., 2009, 2010). However, background strain may influence cellular events and seizure thresholds in mice after TBI (Chrzaszcz et al., 2010). Other studies detected only mild mossy fiber reorganization in posttraumatic mice at similar time points after injury (Hanell et al., 2010). These findings could be due to considerable technical differences among laboratories or high variability in the degree of cortical damage in individual animals. Tissue responses produced after CCI injury depend greatly on external injury parameters (i.e., impact depth

* Corresponding author at: Department of Physiology, University of Kentucky College of Medicine, MS508 Chandler Medical Center, 800 Rose Street, Lexington, KY 40536, USA. Tel.: +1 859 323 4840; fax: +1 859 323 1070.
E-mail address: bret.smith@uky.edu (B.N. Smith).

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doi:10.1016/j.eplepsyres.2011.10.011

and velocity, impactor shape and size, and number of craniotomies) (Mao et al., 2010; Pleasant et al., 2011). While CCI is increasingly used to model epilepsy-related changes after TBI, the parameters of focal cortical damage necessary to consistently reproduce MFS in posttraumatic animals is unknown.

Methods

All procedures were approved by the University of Kentucky Animal Care and Use Committee and adhered to NIH guidelines for the care and use of laboratory animals. Six- to ten-week-old CD-1 (Harlan), C57BL/6 (The Jackson Laboratory), or FVB/N (The Jackson Laboratory) mice were subjected to a unilateral cortical contusion by CCI injury as previously described (Hunt et al., 2009, 2010, 2011). We chose these strains because they display different cellular responses in status epilepticus models (Schauwecker and Steward, 1997), and/or are often used in transgenic studies. Severe brain injury was delivered using an electronically controlled, pneumatically driven impactor fitted with a stainless steel tip 3 mm in diameter (Precision Systems and Instrumentation, Fairfax, VA) to compress the cortex to a depth of 1.0 mm (or, for C57BL/6 mice only, 1.2 mm), at 3.5 m/s and 400—500 ms duration. Mice were injured with two differently shaped impactor tips, beveled or rounded, to achieve a variable degree of cortical damage. Impactor shape is an important determinant for CCI-induced cortical damage (Mao et al., 2010; Pleasant et al., 2011) and has been a common variation among studies examining epilepsy-related changes after CCI. A subset of injured CD-1 mice was monitored for injury-induced behavioral seizures during a 90 min interval beginning 90 min post-injury. Seizure severity was scored from 1 to 5, according to a modified Racine scale (Hunt et al., 2009, 2010).

Mice were perfused with 0.37% sodium sulfide in 0.1 M $NaHPO_4$ followed by 4% paraformaldehyde in 0.15 M phosphate buffer. Brains were cryoprotected with 30% sucrose in 0.01 M phosphate-buffered saline; 20 μm coronal brain sections were cut on a cryostat and collected at 400 μm intervals. Timm's and Nissl staining was performed as previously described to visualize mossy fibers and cell bodies (Shibley and Smith, 2002) ipsilateral and contralateral to the injury. Timm's scores were plotted with respect to the distance of each section from the injury epicenter, which was qualitatively defined as the section with the most extensive cortical damage. Scores for sprouting were assigned from 0 to 3 based on the rating scale of Tauck and Nadler (1985). If Timm's staining between the blades of the granule cell layer was variable, an averaged score was used (e.g., if the lower blade was scored 1 while the upper blade was scored 2, the section was given an overall grade of 1.5). MFS was defined as at least one section with a Timm's score >1 (Hunt et al., 2009, 2010, 2011).

Data were analyzed using Microsoft Excel and Instat3 programs. Numerical data are presented as the mean ± SD. The nonparametric Chi square or Kruskal—Wallis test with Dunn's post hoc tests were used to analyze Timm score differences between groups. Mann—Whitney *U* was used to examine differences between pairs. Significance was set at $P < 0.05$.

Results

Gross damage 8—12 wks after CCI consisted of a cortical cavity 3 mm in diameter extending through the thickness of the neocortex at the injury epicenter, located midway between lambda and bregma, 5 mm lateral to midline. In all mice injured with a rounded-tip impactor, the cortical cavity at the injury site was restricted to the neocortex ($n = 5$ CD-1; $n = 9$ FVB; $n = 12$ C57BL/6). In most mice injured with a beveled tip, a variably sized cavity extended into the hippocampus (260—1070 μm^3), accompanied by hippocampal distortion extending 300—1600 μm from the injury epicenter ($n = 18$ of 20 CD-1; $n = 18$ of 23 FVB; $n = 7$ of 10 C57BL/6). These results are consistent with recent studies demonstrating greater hippocampal damage after injuries administered using beveled versus rounded-tip impactors (Mao et al., 2010; Pleasant et al., 2011).

MFS was detected ipsilateral to the injury in all three strains of posttraumatic mice. In contrast, none of the hippocampi contralateral to the injury had abnormal mossy fiber organization (i.e., all Timm scores were ≤ 1). The degree of hippocampal distortion and pattern of MFS were variable (Fig. 1). The most robust Timm's staining was always found within 800 μm of the injury epicenter toward the ventral pole (Fig. 2).

All mice, regardless of strain, in which the cortical cavity impinged upon the hippocampus had MFS into the inner molecular layer ipsilateral to the injury (CD-1, $n = 18$ of 18; FVB, $n = 18$ of 18; C57BL/6, $n = 7$ of 7). In mice where the cavity was restricted to the neocortex, MFS was observed ipsilateral to the injury in 57% of CD-1 ($n = 4$ of 7), 29% of FVB ($n = 4$ of 14), and 27% of C57BL/6 ($n = 4$ of 15) mice, with no detectable difference between strains ($X^2 = 2.241$, d.f. = 2, $P = 0.33$). We evaluated ''peak'' Timm scores in sections that were 400 μm ventral to the injury epicenter to examine whether damage to the hippocampus was associated with greater MFS. For this analysis, we compared hippocampi ipsilateral to the injury, in mice with and without a cavity into the hippocampus, with contralateral hippocampi. A Kruskal—Wallis test detected a significant difference in Timm score ranges among groups for each strain (CD-1: $H_{(2, 49)} = 38.58$, $P < 0.001$; FVB: $H_{(2, 61)} = 47.21$, $P < 0.001$; C57BL/6: $H_{(2, 43)} = 24.79$, $P < 0.001$; Fig. 2E). Post hoc analysis revealed that ipsilateral hippocampi had higher Timm scores than contralateral hippocampi for all strains, regardless of the extent of cortical damage. However, Timm scores were greater in mice in which the cortical cavity included portions of the hippocampus versus mice in which the cavity was restricted to the neocortex, regardless of strain. No difference was detected in the time post-TBI in which MFS was evaluated between mice with (9.86 ± 1.1 wks) and without (9.22 ± 0.8 wks) a cavity into the hippocampus for any strain ($P > 0.05$).

The development of MFS after pilocarpine administration in mice relates to seizure number induced during status epilepticus (Shibley and Smith, 2002). Therefore, we evaluated whether Timm's scores were greater in CD-1 mice that displayed immediate injury-induced behavioral seizures versus mice that did not have seizures. Five mice displayed immediate seizures after TBI (one to four seizures/mouse; category 2—5) and had an average Timm's score of 2.2 ± 0.27. Mice that did not have immediate seizures had an average Timm's score of 2.2 ± 0.53 ($n = 20$; $P > 0.05$). Immediate seizures after TBI did not predict the development of posttraumatic MFS.

Discussion

Our finding that MFS is increased in mice with posttraumatic hippocampal cavitation is consistent with previous

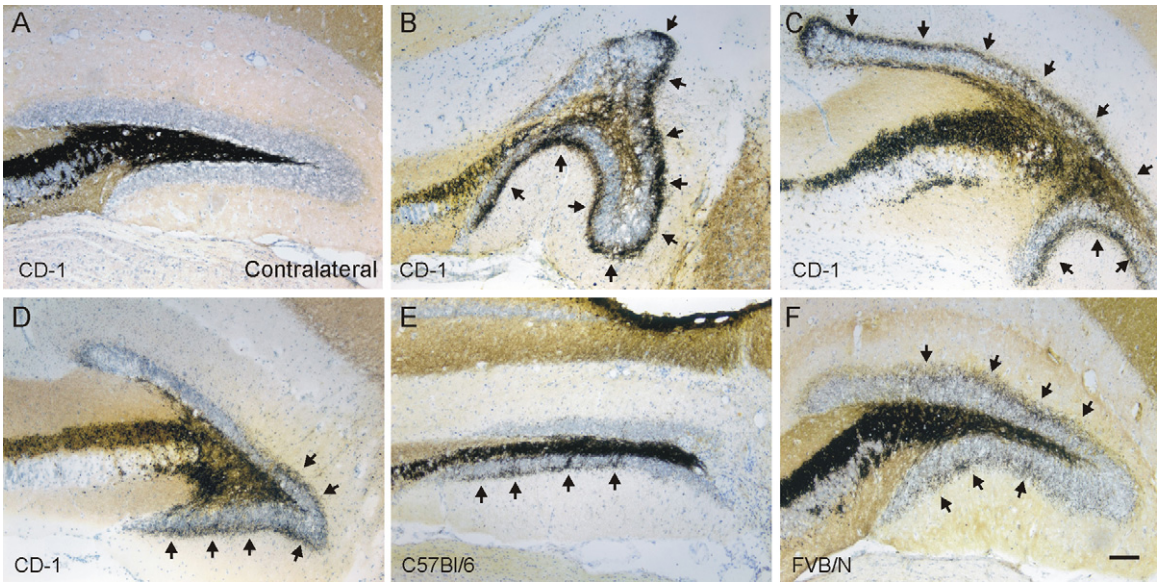

Figure 1 MFS after cortical contusion injury is not uniform. Example Timm's and Nissl stained sections of the dentate gyrus 8—12 wk after CCI injury. (A) Representative image of hippocampus contralateral to the injury shows the absence of mossy fiber sprouting in the inner molecular layer (Timm score = 0). (B—F) Representative images of Timm's staining in the ipsilateral dentate gyrus near the injury epicenter. Note that the pattern of MFS and distortion of the granule cell layer is different in each section. MFS into the inner molecular layer is indicated by arrows. (B—D) Sections obtained from CD-1 mice. Timm scores for these sections are B, 2; C, 3; D, 2. E. Section from a C57BL/6 mouse (Timm score = 1.5). F. Section from an FVB mouse (Timm score = 2). Scale bar is 100 μm.

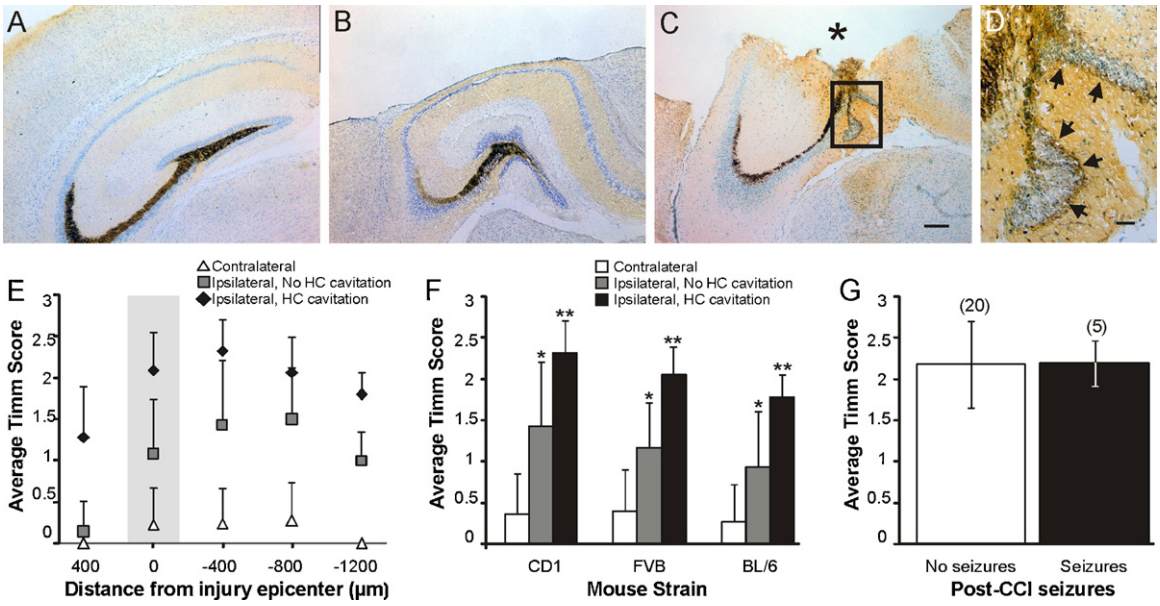

Figure 2 Timm scores are greater in mice with cortical cavitation that enters the hippocampus (HC). (A) Image of Timm's and Nissl stained contralateral dentate gyrus. (B) Image of Timm's and Nissl stained ipsilateral dentate gyrus at the injury epicenter. Note that the cortical cavity does not include portions of the hippocampus. (C) Image of Timm's and Nissl stained ipsilateral dentate gyrus at the injury epicenter in an animal where the cortical cavity extended into the hippocampus (asterisk). The contralateral hippocampus from this mouse is shown in (A). (D) Enlarged image of the boxed area in (C) shows MFS into the inner molecular layer (arrows). Scale bar is 200 μm in (A—C) and 50 μm in (D); sections from CD-1 mice shown in (A—D). (E) In CD-1 mice, average Timm score in relation to the distance from the injury epicenter (zero on the *x*-axis), septal (400 μm) to temporal (−1200 μm). (F) Average ''peak'' Timm score at −400 μm for each group in CD-1, FVB, and C57BL/6 (BL/6) mice. Asterisk indicates significant difference from slices contralateral to the injury. Double asterisk indicates significant difference from both contralateral hippocampi and ipsilateral hippocampi without hippocampal cavity. (G) Timm scores 8—12 weeks after injury are not greater in mice observed to have behavioral seizures in the first 90 min post-TBI, versus mice in which seizures were not observed. Number of mice in each category is shown in parentheses.

reports describing greater spontaneous seizure incidence after severe CCI using a beveled impactor (36—40%; Hunt et al., 2009, 2010) versus rounded-tip impactors (9—13%; Bolkvadze et al., 2009; Statler et al., 2009). Hippocampal damage was more likely with beveled tips. Injuries without hippocampal cavitation resulted in less prevalent MFS, despite similar impact depth. In addition to impact parameters, rodent species, animal age, or injury location, might also affect MFS and seizure incidence after CCI injury.

The relatively low seizure incidence in PTE models suggests the need for surrogate biomarkers. We found that the degree of neocortical damage might be a less useful predictor of posttraumatic MFS than is hippocampal cavitation; all mice with hippocampal cavitation developed sprouting. Why MFS occurs is controversial. Among potential triggers include hilar or hippocampal cell loss, neurogenesis, and growth factor overexpression, all of which occur after CCI. Correlation of MFS with these parameters may be useful for identifying other key features of posttraumatic epileptogenesis. While MFS is qualitatively related to epileptogenesis, it does not correlate quantitatively with seizure frequency or severity in temporal lobe epilepsy models (Buckmaster and Dudek, 1997). MFS ipsilateral to TBI might be related to posttraumatic EEG spike activity (Kharatishvili et al., 2007); MRI markers have been used to evaluate brain damage after brain injury in rodents (Kharatishvili et al., 2007, 2009; Onyszchuk et al., 2007). Perhaps the presence of hippocampal damage could serve as a biomarker for animals with the highest probability for developing epilepsy.

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$GABA_A$ Receptor Regulation after Experimental Traumatic Brain Injury

Daniel J. Raible[1], Lauren C. Frey[2], Yasmin Cruz Del Angel[4], Shelley J. Russek[3], and Amy R. Brooks-Kayal[1, 2,4,5]

[1]Neuroscience Program, University of Colorado, Anschutz Medical Campus, Aurora, Colorado.

[2]Department of Neurology, University of Colorado School of Medicine, Aurora, Colorado.

[3]Laboratory of Translational Epilepsy, Department of Pharmacology, Boston University School of Medicine, Boston, Massachusetts.

[4]Division of Neurology, Department of Pediatrics, University of Colorado School of Medicine, Aurora, Colorado.

[5]Children's Hospital Colorado, Aurora, Colorado.

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Corresponding Author: Amy R. Brooks-Kayal

Author Contact Information:

1. Daniel J. Raible, BS
   University of Colorado AMC
   Department of Pediatrics
   12850 E. Montview BLVD C238
   RM 3440D School of Pharmacy
   Aurora CO 80045-2605
   Phone: 480.628.3257
   Fax: (303) 724-5466
   Email. Daniel.raible@ucdenver.edu

2. Lauren C. Frey, MD
   University of Colorado AMC
   Department of Neurology
   Mailstop B-182
   12700 East Nineteenth Avenue
   Aurora, CO 80045
   Phone: 303.724.2199
   Fax: 303.724.4329
   Email. Lauren.Frey@ucdenver.edu

3. Yasmin Cruz Del Angel BS
University of Colorado AMC
Department of Pediatrics
12850 E. Montview BLVD C238
RM 3440D School of Pharmacy
Aurora CO 80045-2605
Phone: 303.724.2439
Fax: 303.724-5466
Email: Yasmin.cruzdelangel@ucdenver.edu

4. Shelley J. Russek PhD
Boston University School of Medicine
Department of Pharmacology
72 East Concord St. L-603
Boston, MA 02118
Phone: 617.638.4319
Fax: 617.638.4329
Email: srussek@bu.edu

5. Amy R. Brooks-Kayal MD *Corresponding author
University of Colorado AMC
Department of Pediatrics
12850 E. Montview BLVD C238
RM 3440D School of Pharmacy
Aurora CO 80045-2605
Phone: 303.724.2439
Fax: 303.724.5466
Email: Amy.Brooks-Kayal@childrenscolorado.org

Abstract

The γ-aminobutyric acid (GABA) type A receptor ($GABA_AR$) is responsible for most fast synaptic inhibition in the adult brain. The $GABA_AR$ protein is composed of multiple subunits, which determine the distribution, properties and dynamics of the receptor. Several studies have shown that the Janus Kinase / Signal Transducer and Activator of Transcription (JaK/STAT) and Early growth response 3 (Egr3) signaling pathways can alter $GABA_AR$ subunit expression after status epilepticus (SE). This study investigated changes in these pathways after experimental TBI in the rat using a lateral fluid percussion injury (FPI) model. Our results demonstrated changes in expression of several $GABA_AR$ subunit levels after injury including $GABA_AR$ α1 and α4 subunits. This change appears to be transcriptional and there is an associated increase in the phosphorylation of STAT3 and an increase in the expression of Egr3 and ICER after FPI. These findings suggest that the activation of the JaK/STAT and Egr3 pathways after TBI may regulate injury-related changes in $GABA_AR$ subunit expression.

Keywords: Traumatic brain injury**,** $GABA_A$ receptor, JaK/STAT pathway and Egr3 pathway.

Introduction

Traumatic brain injury (TBI) is a major public health problem worldwide and has been called the signature injury of the US conflicts in Iraq and Afghanistan. Each year, TBI is a leading cause of mortality and neurological morbidity (Sosin et al., 1996, Thurman et al., 1999). In addition, up to 50% of TBI survivors can go on to develop epilepsy (Salazar et al., 1985, Marcikic et al., 1998). A subsection of these develop temporal lobe epilepsy (TLE) (Diaz-Arrastia et al., 2000, Hudak et al., 2004).

Preventative and therapeutic treatments for many of these patients have been unsuccessful due to the lack of knowledge of the cellular and molecular mechanisms by which brain trauma causes TLE. This lack of understanding limits how current medications are being implemented and designed.

Trauma to the brain initiates a complex sequence of molecular responses involving a number of signaling pathways. One such pathway is the Janus Kinase / Signal Transducer and Activator of Transcription (JaK/STAT) pathway, which has been shown to be activated after TBI (Oliva et al., 2011, Zhao et al., 2011a, Zhao et al., 2011b) and following other brain insults such as stroke (Planas et al., 1996, Suzuki et al., 2001) and status epilepticus (SE) (Choi et al., 2003, Lund et al., 2008). Following SE, the JaK/STAT pathway has been shown to regulate the γ-aminobutyric acid (GABA) type A receptor ($GABA_AR$) subunit α1 expression in the hippocampus by increasing the phosphorylation of STAT3 (Lund et al., 2008). Phosphorylated STAT3 (pSTAT3) subsequently decreases transcription of the $GABA_AR$ α1 gene (*Gabra1*) by increasing the expression of the cyclic AMP response element-binding protein (CREB) family transcriptional inhibitor inducible cAMP element repressor (ICER) (Lund et al., 2008). ICER binds to the *Gabra1* promoter with phosphorylated CREB (pCREB) to inhibit the expression of *Gabra1* (Figure 1) (Lund et al., 2008). The decreased levels of α1 subunit-containing $GABA_AR$ are thought to contribute to the increased hyperexcitability seen in the hippocampus of animals following SE and to contribute to subsequent development of TLE (Brooks-Kayal et al., 1998, Peng et al., 2004, Zhang et al., 2004, Raol et al., 2006). Decreases in the $GABA_AR$ α1 subunit have been reported in injured hippocampus after TBI (Gibson et al., 2010), but whether the JaK/STAT pathway regulates this

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decrease is not known. Other $GABA_AR$ subunits are also modulated after cerebral insults such as SE (Brooks-Kayal et al., 1998, Peng et al., 2004, Sun et al., 2007, Zhang et al., 2007), and may also contribute to the alterations of neural excitability.

Lateral fluid percussion injury (FPI) in the rat was used for our studies to determine if there were alterations acutely in $GABA_AR$ subunit levels and the known signaling pathways which alter these receptor levels after other types of cerebral injury (such as SE). Lowenstein et al. (1992), Toth et al. (1997) and Gupta et al. (2012) have shown that as early as 1 week after FPI there is enhanced excitability in the hippocampus which indicates that TBI alters the balance of excitation and inhibition early after the injury (Lowenstein et al., 1992, Toth et al., 1997, Gupta et al., 2012). D'Ambrosio et al. (2004) and Kharatishvili et al. (2006) have shown that these animals can go on to develop epilepsy (D'Ambrosio et al., 2004, Kharatishvili et al., 2006). The molecular mechanisms mediating early changes in excitability after TBI as well as the contribution of early molecular and electrophysiological changes to later development of post-traumatic epilepsy (PTE) are not fully understood.

In this paper, we examined the levels of $GABA_AR$ subunits after lateral fluid percussion injury (FPI) in the rat and found that the α1, α4, γ2 and δ subunit levels are decreased 1 week after injury and that α2 and α5 subunit levels remain unchanged. We also found that two pathways known to regulate $GABA_AR$ subunit composition, the JaK/STAT and Egr3 pathways, are activated after FPI.

Materials and Methods

Establishment of dural access and fluid percussion injury

Adult male Sprague-Dawley rats (250-300g) were anesthetized with 3-5% isoflurane via nose cone and placed in a stereotaxic head frame. After scalp incision and reflection, a 3-mm diameter craniotomy was created and centered at -3 mm from bregma and 3.5 mm left of the sagittal suture. For support, a steel screw was placed in the right parietal bone opposite the craniotomy site. A female Luer-Loc hub was centered over the craniotomy site and bonded to the skull with cyanoacrylate adhesive. Dental acrylic was poured around the Luer hub and support screw. After the acrylic hardened, antibiotic ointment was placed around the injury cap and the animal was removed from the stereotaxic frame and returned to his cage to recover.

Fifteen to 20 hours after craniotomy and Luer hub implantation, the animals were anesthetized with isoflurane in an induction chamber. The animal was then removed from the chamber, immediately connected to the FPI apparatus, and received a 20 ms pulse of pressurized fluid (2.5-3.0 atm, moderate to severe impact) on the intact dural surface before awakening from anesthesia (Frey et al., 2009). Sham injured animals underwent establishment of dural access and were anesthetized and connected to the FPI apparatus, but the injury pulse was not triggered. A total of 81 animals underwent surgery of which 34 were sham injured animals and 47 were FPI animals. Out of the animals that underwent FPI 37 animals lived, thus we had a survival rate of 79%. The University of Colorado Institutional Animal Care and Use Committee approved all procedures described.

After the FPI the animals were returned to their cage and allowed to recover for 6 hours, 24 hours, 48 hours, or 1 week after injury. At the above time points, animals were deeply anesthetized with isoflurane in an induction chamber, decapitated, and their brains

were rapidly removed. The hippocampi were dissected and flash frozen for storage in a -80° C freezer.

Western blot

Western blot was performed with modifications of published protocols (Raol, et al., 2006). Protein (25 μg) extracted from whole hippocampus was loaded into 8% SDS-polyacrylamide gels and run for 1.5 hours at 115 V. Blots were then transferred to nitrocellulose membranes and blocked in 5% milk/trisphosphate-buffered saline with Tween-20 (TBS-T). Membranes were incubated with rabbit polyclonal antibodies raised against STAT3 phosphorylated at Tyr705 (anti-pSTAT3) (Cell Signaling Technologies; 1:1000), STAT3 (Cell Signaling Technologies; 1:2000), α1 (Millipore; 1:5000), α2 (Millipore, 2,000), α4 (Millipore; 2,000), α5 (Millipore; 1:1,000), δ (Millipore; 1:1,000), or β actin (Sigma 1:40,000) overnight at 4°C in 5% bovine serum albumin/ TBS-T (for pSTAT3 and STAT3) or 1% milk/TBS-T (for $GABA_AR$), then washed and incubated with anti-rabbit antibody (1:10,000) conjugated to horseradish peroxidase (HRP) for 1 hour. Protein bands were detected with the use of chemiluminescent solution (Pierce), then membranes were stripped and reprobed with rabbit polyclonal antibody raised against total STAT3 (anti-STAT3) (Cell Signaling Technologies; 1:2000) or β-actin and bands were quantified using Image J software (NIH) and expressed as percent change with respect to mean control values in the same run (defined as 100).

RT-PCR

RNA was extracted from whole hippocampus with the use of Trizol reagent protocol (Invitrogen, Carlsbad, CA). To synthesize complementary DNA (cDNA), 1 μg of RNA was separated and processed with the SuperScript II reverse transcription kit

(Invitrogen) according to the manufacturer's instructions and then diluted 1:4 for storage and subsequent RT-PCR. For RT-PCR reactions, each sample was run in triplicate and each 25-µl reaction contained 1.25 µl ICER, Egr3, $GABA_AR$ subunit α1, $GABA_AR$ subunit α4 or cyclophilin (ppia) Taqman gene expression primer/probe sets from Applied Biosystems (Foster City, CA), 12.5 µl of Taqman Master mix, and 10 µl of sample cDNA. RT-PCR formed on the SDS-7500 PCR machine (Applied Biosystems). The RT-PCR runs consisted of 1 cycle of 50°C for 2 min, then 1 cycle of 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. All values were normalized to cyclophilin expression in the same samples to control for loading variability, then expressed as fold change with respect to mean controls values in the same run (defined as 1).

Statistical Analysis

Statistical significance was defined as a P value of less than 0.05. All calculations were done using InStat software. Mean amounts of pSTAT3 and each $GABA_AR$ subunit protein assessed using western blotting were compared between injured and sham injured groups using student t-testing.

Mean RT-PCR expression of α1, α4, ICER and Egr3 were compared between injured and sham injured controls for the ipsilateral and contralateral brain regions (relative to FPI) using student t-testing, or the Mann-Whitney test, as appropriate for non-parametric data.

Results

$GABA_AR$ subunit levels change after FPI

Figure 2 shows $GABA_AR$ subunit protein levels in hippocampus in sham injured and FPI animals. Protein levels of the α1 subunit showed a significant decrease at 24

hours, 48 hours and 1 week post injury in the ipsilateral hippocampus (Figure 2A and B). As shown in Figure 2C and D, α4 subunit levels were significantly increased at 24 hours after injury, were not significantly different from controls at 48 hours and significantly decreased when compared to sham injured controls at 1 week after injury. There was no significant difference in the α1 and α4 subunit levels at any time point in the hippocampus contralateral to the FPI when compared to sham injured controls (data not shown). To investigate whether the α1 and α4 subunit levels changes at 24 hours post injury were due to transcriptional regulation we used quantitative RT-PCR on whole hippocampus samples 6 hours after injury to determine the mRNA levels for these subunits. Figure 3 shows that in the ipsilateral hippocampus 6 hours after FPI there was decreased expression for the α1 subunit (0.4 fold relative to control) and an increase in the α4 subunit expression (1.7 fold relative to control) (Figure 3A and B). There was no statistical difference in mRNA levels in the contralateral hippocampus when compared to sham injured controls (data not shown). Figure 4 shows that γ2 and the δ subunit levels are significantly decreased 1 week after injury, but that the α2 and α5 are unchanged when compared to sham injured controls. There was no significant difference in the γ2, δ, α2 or α5 subunit levels in the contralateral hippocampus when compared to sham injured controls (data not shown).

FPI transiently activates the JaK/STAT pathway and affects downstream expression of ICER

As shown in Figure 5A and B, there is a statistically significant increase in the levels of pSTAT3 in the ipsilateral hippocampus when compared to sham injured controls at 6 hours after injury. This increase declines temporally and at one week there is no difference in the levels of pSTAT3 when compared to controls. Figure 5C and D shows

that the contralateral hippocampus there was a statistically significant increase in the pSTAT3 levels 6 hours after injury but at 24 hours after FPI the levels were not statistically different from sham injured controls. This suggests that the pathway is transiently being activated after FPI.

Figure 6A shows that ICER mRNA levels in the hippocampal ipsilateral to the injury are significantly increased 6 hours after injury, compared to sham injured controls, although there is no difference between groups in the contralateral hippocampus (data not shown).

FPI increases Egr3 levels

The Egr3 pathway has been shown to be a critical mediator of α4 up regulation after SE (Roberts et al., 2006). Figure 6B shows that Egr3 mRNA levels in the hippocampal ipsilateral to injury are significantly increased at 6 hours after injury, compared to sham injured controls, although there is no difference between groups in the contralateral hippocampus (data not shown).

Discussion

In this study, we demonstrated that hippocampal injury after FPI alters the levels of protein and mRNA of several $GABA_AR$ subunits, activates the JaK/STAT pathway, and elevates Egr3 mRNA levels. The $GABA_AR$ α1subunit level is decreased 24 hours after injury and remains lower than sham injured controls for at least 1 week after FPI. The α4 subunit level is increased 24 hours after injury and then is lower than sham injured controls by one week post-injury. We also found that mRNA expression of the α1subunit was statistically decreased and that of the α4 subunit mRNA was statistically increased at 6 hours after FPI, suggesting that the changes in the $GABA_AR$ subunit levels are at least

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in part due to transcriptional regulation.

Several other $GABA_AR$ subunits were analyzed to determine if their levels also changed after injury. Our goal for looking at multiple $GABA_AR$ subunits was to determine if there was an overall decrease in all subunits at one week or if there was evidence to suggest subunit specific regulatory processes occurring after FPI. Further, in some transgenic mice with $GABA_AR$ subunit mutations, as well as after SE in the rat, it has been shown that changes in expression of one $GABA_AR$ subunit can be associated with unanticipated regulation of other subunits (Brooks-Kayal et al., 1998, Sur et al., 2001, Kralic et al., 2006, Schneider Gasser et al., 2007, Liang et al., 2008). While the γ2 and δ subunit levels were found to decrease at one-week post injury, the α2 and α5 subunit levels showed no significant difference between FPI animals and sham injured controls at one week post-injury. These findings suggest that subunit changes are in part due to subunit specific regulatory processes.

A recent study using FPI in the rat found a reduction of approximately 55% of $GABA_AR$ α1 subunit levels compared to sham injured controls with no change in α2 and α5 subunits in the ipsilateral hippocampus after 24 hours and 1 week after injury, similar to our results (Gibson et al., 2010). These authors did not examine the α4 subunit or any of the signaling pathways which regulate $GABA_AR$ subunits. Another study using the FPI model in the rat showed that after experimental TBI there is a progressive loss of phasic $GABA_AR$ mediated inhibition, which is suggestive of alterations of the $GABA_AR$. This study, however, only showed $GABA_AR$ subunit mRNA expression changes of α4 (a ~52% increase expression compared to control) and β1 (a ~27% increase in expression compared to control) (Pavlov et al., 2011). The difference in the expression levels seen

between this study and ours could be attributed to when the samples were collected, which was 6 hours after injury in the current study while the Pavlov et al. (2011) measured $GABA_AR$ mRNA levels 4 months after injury (Pavlov et al., 2011).

Studies utilizing another injury model, controlled cortical impact (CCI) in the rat, showed a reduction in γ2 subunit levels ~5-9 months after injury similar to those found in the current study but showed no change in α1 and α4 and an increase in δ (Kharlamov et al., 2011). These differences may be attributed to the injury methods (CCI vs. FPI), the time point of collecting the samples, and that Kharlamov, et al. (2011), only analyzed the contralateral hippocampus, a brain region not known to be extensively affected in the CCI model. Electrophysiological studies done using the same injury model found that there is an increase in the $GABA_AR$-mediated tonic currents, a loss of diazepam potentiation and an increase in furosemide inhibition of synaptic $GABA_ARs$ in dentate granule cells (DGCs) ipsilateral to the injury site 90 days after injury (Mtchedlishvili et al., 2010). These physiological changes are consistent with a decrease in α1 containing receptors and an increase in α4 containing receptors in DGCs similar to that seen in rats with epilepsy following SE (Brooks-Kayal et al., 1998). In addition, the γ2 and δ subunit levels have been shown to influence tonic and phasic synaptic inhibition and $GABA_AR$ location (Somogyi et al., 1996, Nusser et al., 1998, Sur et al., 1999, Sassoe-Pognetto et al., 2000, Wei et al., 2003, Semyanov et al., 2004, Sun et al., 2004, Jia et al., 2005, Liang et al., 2006, Zhang et al., 2007, Goodkin et al., 2008). Alteration of all of these levels, which we also see after FPI, may result in altered GABAergic signaling and could be a contributing factor in the development of TLE after head trauma.

Previous studies have shown that the JaK/STAT pathway mediates a decrease in α1

subunit levels after SE (Brooks-Kayal et al., 1998, Lund et al., 2008). SE results in phosphorylation of STAT3, which upregulates expression of ICER, and subsequently binds to the *Gabra1* promoter and decreases transcription of *Gabra1* in the hippocampus of rats (Lund et al., 2008). Multiple types of injuries to the hippocampus can lead to the development of TLE (including SE, ischemia and head trauma). Interestingly, the JaK/STAT pathway activation has been shown to be activated in each of these types of injury models (Planas et al., 1996, Suzuki et al., 2001, Lund et al., 2008, Oliva et al., 2011, Zhao et al., 2011a, Zhao et al., 2011b).

In the current study, we found that pSTAT3 levels were significantly increased in the injured hippocampus 6 hours after FPI and that there is a subsequent decline in pSTAT3 levels back to baseline levels by one week after injury. Our data is consistent with data from Oliva et al. (2011) suggesting that the JaK/STAT pathway is transiently activated in the ipsilateral hippocampus after cerebral injury (Oliva et al., 2011). Oliva et al. (2011) also showed pSTAT3 co-localizing mostly with GFAP positive cells and that pSTAT3 was transcriptionally regulating known STAT3 regulatory genes such as nitric oxide synthase 2 (Oliva et al., 2011). However, they did not look for alterations in $GABA_AR$ genes, subunit levels, or ICER or Egr3 expression. Zhao et al. (2011) performed western blot analysis and immunohistochemistry which showed that in the injured ipsilateral parietal cortex pSTAT3 signal was enhanced at acute time points and that pSTAT3 was co-localized with both NeuN and GFAP positive cells after TBI (Zhao et al., 2011b). It still needs to be determined if the activation of the JaK/STAT pathway after TBI directly alters the expression of $GABA_AR$ subunits as has been shown after SE, and is a contributing factor in the development of TLE after TBI.

Lastly, we looked at the Egr3 mRNA levels 6 hours after FPI because the α4 subunit was increased 24 hours after injury and previous studies in our lab have shown that Egr3 pathway can regulate transcription of the α4 subunit (Roberts et al., 2005, Roberts et al., 2006, Gangisetty and Reddy, 2010). We have shown that the Egr3 mRNA levels were increased in the ipsilateral hippocampus after TBI, suggesting that this pathway may be regulating the increase in the α4 subunit levels 24 hours after injury. The mechanism mediating the reduction in α4 subunit levels 1 week post injury remains uncertain. Future studies will be required to elucidate these mechanisms as well as to understand the functional role of Egr3 changes after FPI (Gangisetty and Reddy, 2010).

Our study does have several limitations. First, the whole hippocampus was used to determine the $GABA_AR$ subunit levels as well as for the determination of pSTAT3 levels and expression of ICER and Egr3. Thus, we are unable to determine where in the hippocampus these changes were occurring and in which cell type. Second, β-actin was used for normalization in this study to control for differences in protein loading, and β-actin levels can be affected by cell death or other regulatory processes. However, normalization of subunit densitometry to total protein loaded in each well produced results similar to those found with normalization to β-actin and there was no difference in the expression of β-actin (normalized to total protein loaded) in the FPI samples compared to sham injured controls (data not shown). Thus, we could find no evidence that intergroup differences in β-actin levels were impacting our results. Further, we could find no studies in the literature suggesting that β-actin is actively regulated after FPI. Third, our data suggests association, but not necessarily a causal relationship between activation of the JaK/STAT and Egr3 pathways and $GABA_AR$ alterations. A causal relationship has been shown more definitively in TLE after SE (Roberts et al., 2005, Roberts et al., 2006, Lund et al., 2008, Gangisetty and Reddy, 2010), suggesting that a causal relationship between the two processes in TLE after TBI is plausible, if yet unproven. Fourth, we have only reported a set of molecular

Mary Ann Liebert, Inc, 140 Huguenot Street, New Rochelle, NY 10801

changes after TBI, but do not examine potential functional consequences of these changes. Results of previous studies examining inhibitory function utilizing similar injury models, however, are consistent with changes that we show (Mtchedlishvili et al., 2010, Pavlov et al., 2011). Lastly, while the current findings demonstrate changes in $GABA_AR$s after TBI, they do not establish that these changes contribute to the later development of epilepsy. Establishing this connection will require future studies in which $GABA_AR$ changes after TBI are prevented or reversed and showing that subsequent epilepsy outcome is altered.

Conclusions

The current findings demonstrate that levels of the α1, α4, γ2 and δ $GABA_AR$ subunits are decreased in injured hippocampus at 1 week after experimental TBI and that α2 and α5 subunit levels remain unchanged, suggesting that there is subunit specific regulation of $GABA_AR$s following TBI. We also found that mRNA expression of the α1subunit was decreased and that α4 subunit mRNA was increased, suggesting that the changes in these $GABA_AR$ subunits are at least in part due to transcriptional regulation. Lastly, we found that two pathways known to regulate $GABA_AR$ subunit composition in other models of cerebral injury, the JaK/STAT and Egr3 pathways are activated after FPI. Based on evidence from these other models, we can speculate that the activation of these pathways may mediate altered levels of $GABA_AR$ subunits and may contribute to the early hippocampal hyperexcitability previously reported after TBI as well as potentially to the development of later epilepsy. Future studies will need to be done to determine if there is a causal relationship between JaK/STAT activation, Egr3 elevations, and changes in $GABA_AR$ subunit composition, as well as to determine the contribution of these injury-related changes to the occurrence of experimental post-traumatic epilepsy.

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Author Disclosure Statement

No conflicting financial interests exist.

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Figure 1: Overview of signaling pathways activated after SE that contribute to $GABA_AR$ subunit regulation.

Figure 2: Temporal profile of $GABA_A$ α1 and α4 receptor subunits after FPI. (A) Representative western blots from whole hippocampus of rats 24 hours, 48 hours and 1 week after FPI probed with anti-$GABA_AR$ α1 and β-actin antibodies. (B) Quantification of α1 blots showed a significant decrease at 24 hours through 1 week after FPI injury relative to controls. α1 levels were normalized to β-actin levels and expressed as a percent change compared to sham injured controls. (C) Representative western blots from whole hippocampus of rats 24 hours, 48 hours and 1 week after FPI probed with anti-$GABA_AR$ α4 and β-actin antibodies. (D) Quantification of α4 blots showed a significant increase at 24 hours after FPI but at 1 week there is a significant decrease relative to controls. α4 levels were normalized to β-actin levels and expressed as a percent change compared to shams. (24 hours n = 7 for sham, 6 for FPI; 48 hours n = 6 for sham, 8 for FPI; 1 week n = 7 for sham, 7 for FPI).

Figure 3: α1 expression is decreased and α4 expression is increased in injured hippocampus 6 hours after FPI. mRNA levels of α1 and α4 were quantified using RT-PCR analysis and represented as histograms showing the fold change of α1 (A) and α4 (B) 6 hours after FPI and sham injured controls. α1 and α4 mRNA levels were normalized to cyclophilin mRNA levels in the same samples and expressed as fold change compared to shams (defined as 1). * $P < 0.05$ (n = 4 for sham, and 4 for FPI).

Figure 4: $GABA_A$ receptor subunit γ2 is decreased while α2 and α5 are unchanged 1 week after FPI. (A) Representative western blots from whole hippocampus of rats 1 week after FPI probed with anti-$GABA_AR$ γ2 and β-actin antibodies. (B) Quantification of γ2 blots shows that the γ2 subunit is significantly decreased 1 week after FPI relative to sham injured controls. (C) Representative western blots from whole hippocampus of rats 1 week after FPI probed with anti-$GABA_AR$ δ and β-actin antibodies. (D) Quantification of δ blots shows that the δ subunit is significantly decreased 1 week after FPI relative to sham. (E) Representative western blots from whole hippocampus of rats 1 week after FPI probed with anti-$GABA_AR$ α2 and β-actin antibodies. (F) Quantification of α2 blots shows that the α2 subunit is unchanged 1 week after FPI relative to sham. (G) Representative western blots from whole hippocampus of rats 1 week after FPI probed with anti-$GABA_AR$ α5 and β-actin antibodies. (H) Quantification of α5 blots shows that the α5 subunits are unchanged 1 week after FPI. GABAAR subunit levels were normalized to β-actin levels and expressed as a percent change compared to shams. * $P < 0.001$ ($n = 7$ for sham and 7 for FPI).

Figure 5: Phosphorylated STAT3 levels in injured hippocampus after FPI. (A) Representative western blots of protein homogenates from ipsilateral whole hippocampus (relative to FPI) of rats 6 hours, 24 hours, 48 hours and 1 week after FPI probed with pSTAT3 and STAT3 antibodies. (B) Quantification of pSTAT3 levels from FPI and sham injured controls. (C) Representative western blots of protein homogenates from contralateral whole hippocampus (relative to FPI) of rats 6 hours, 24 hours, 48 hours and 1 week after FPI probed with pSTAT3 and STAT3 antibodies. (D) Quantification of pSTAT3 levels from FPI and sham injured controls. pSTAT3 levels were normalized to STAT3 levels and expressed as a percent change

compared to shams. * $P < 0.05$, ** $P < 0.001$ when comparing sham and FPI animals at the same time point (6 hours n = 5 for sham, 7 for FPI; 24 hours n = 7 for sham, 6 for FPI; 48 hours n = 6 for sham, 8 for FPI; 1 week n = 7 for sham, 7 for FPI).

Figure 6: ICER and Egr3 expression are increased in injured hippocampus 6 hours after FPI. mRNA levels of ICER and Egr3 were quantified using RT-PCR analysis and represented as histograms showing the fold change of ICER (A) and Egr3 (B) 6 hours after FPI in injured and sham injured controls. ICER and Egr3 mRNA levels were normalized to cyclophilin mRNA levels in the same samples and expressed as fold change compared to shams (defined as 1). * $P < 0.05$ (n = 4 for sham, and 4 for FPI).

Figure 1

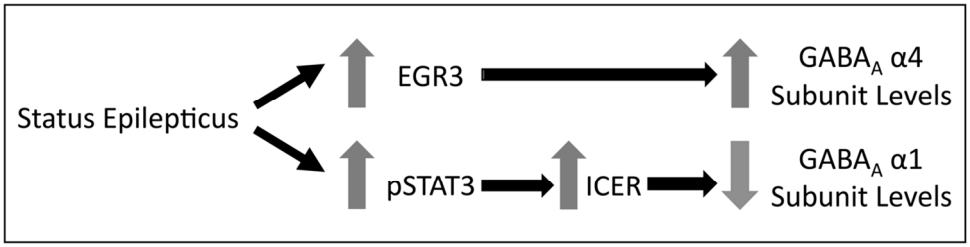

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Figure 2

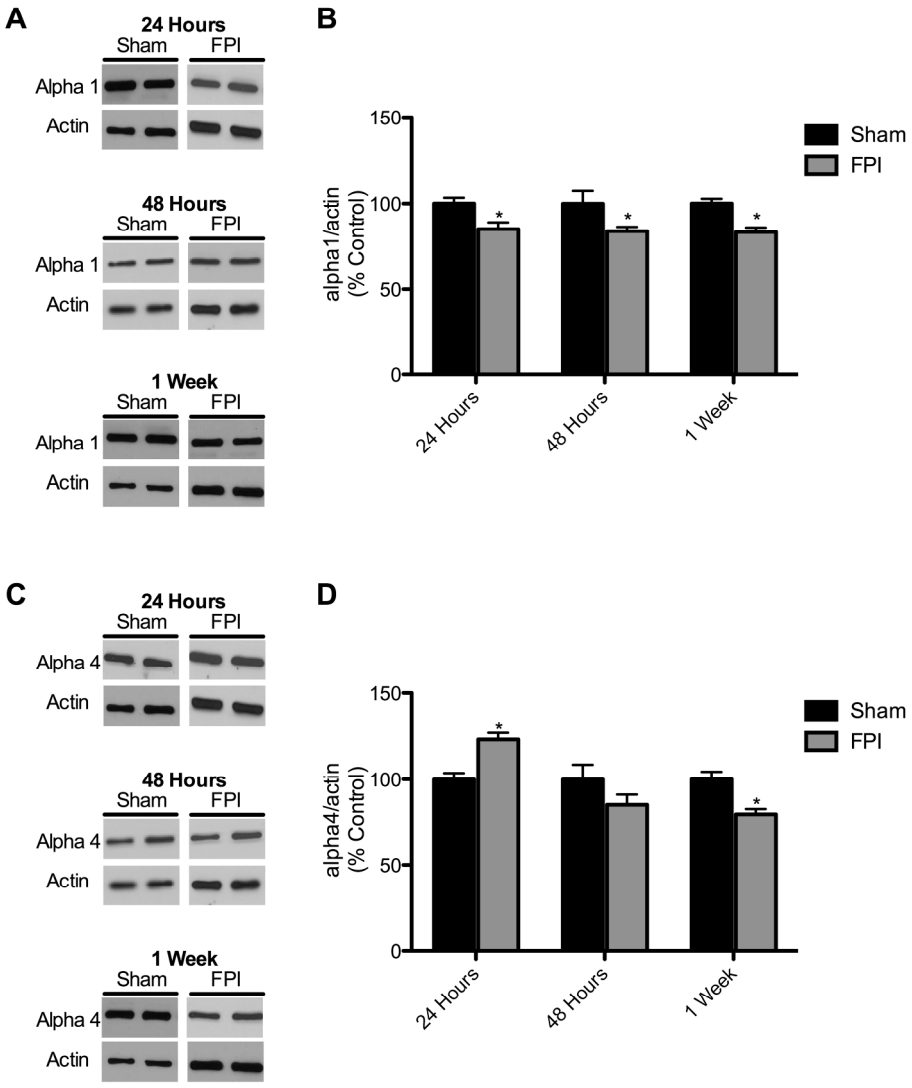

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Figure 3

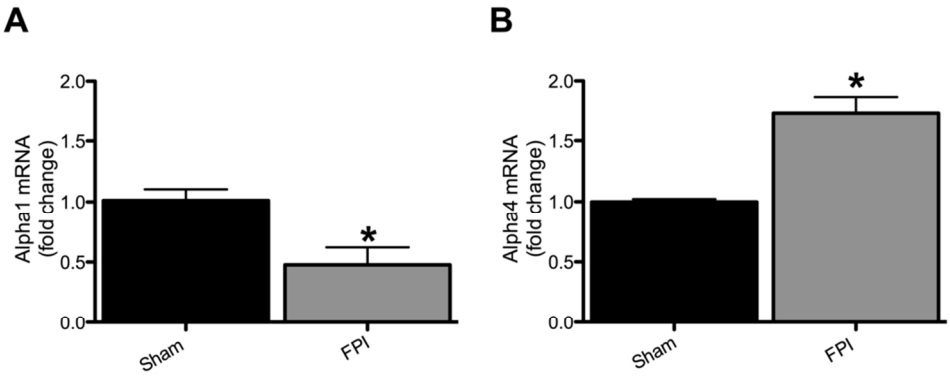

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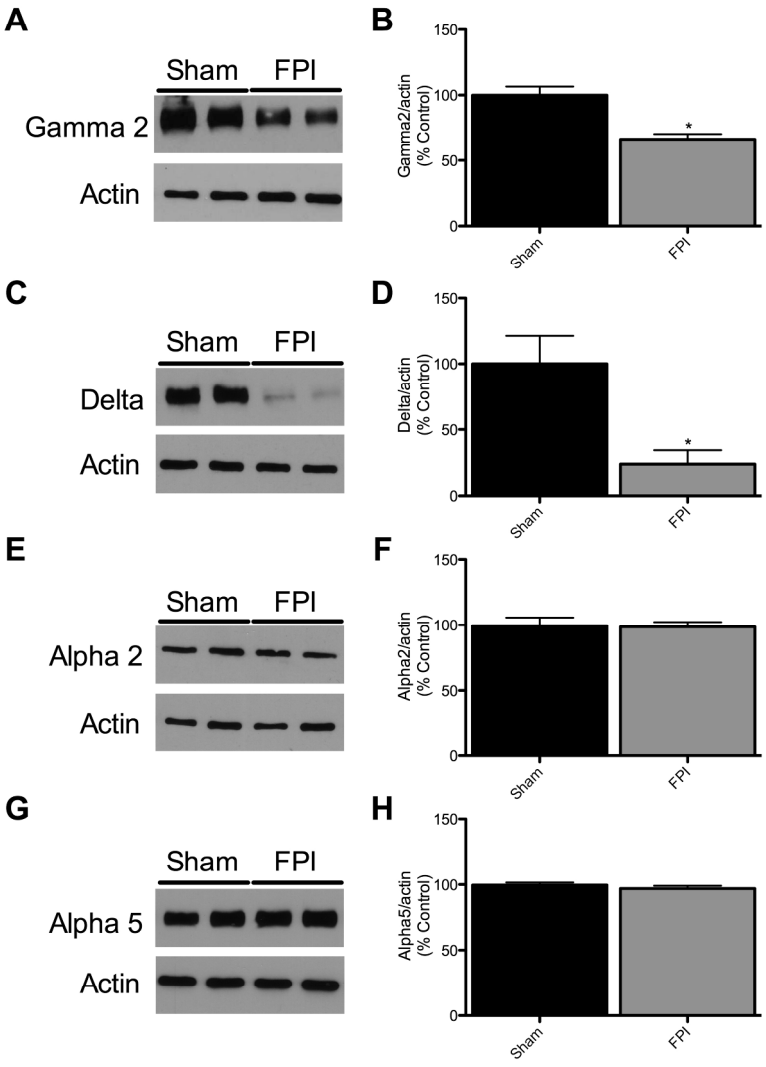
Figure 4



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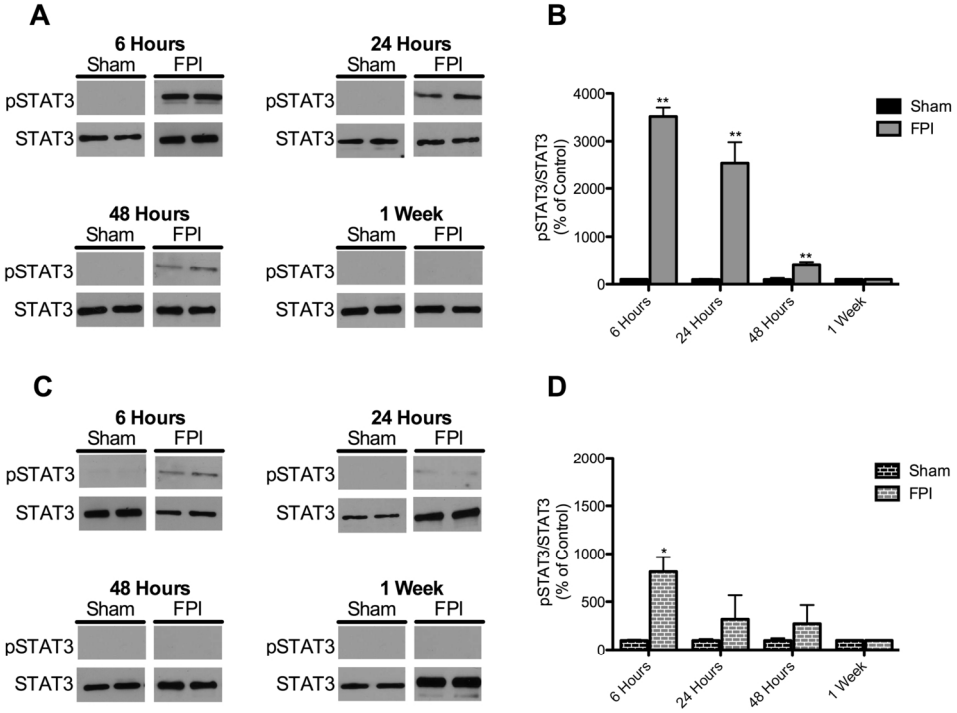

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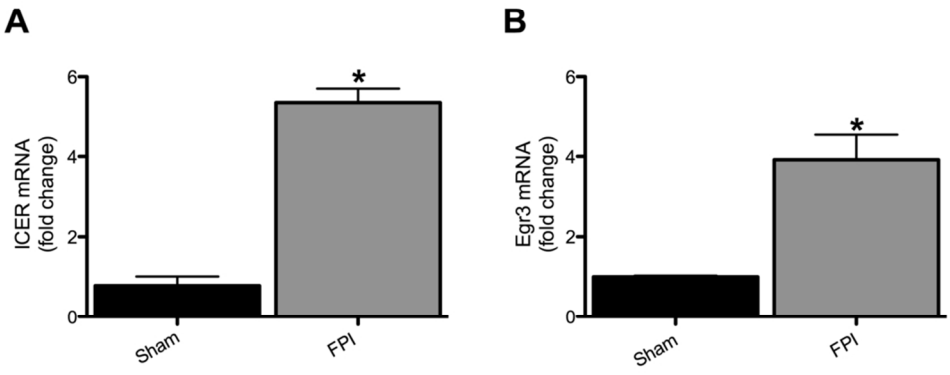
Figure 6



94x48mm (300 x 300 DPI)

JAK/STAT Activation and GABA Neuron Loss After Focal Traumatic Brain Injury in Mice

Butler, CR[1], Boychuk, JA[1], Raible, D[2]., Frey, L.[2], Brooks-Kayal, A.R.[2], Smith, BN[1,3]

[1]Department of Physiology, College of Medicine, University of Kentucky, Lexington, KY 40536
[2]Neuroscience Graduate Program and Departments of Neurology and Pediatrics, University of Colorado Denver, Aurora, CO 80045
[3]Spinal Cord and Brain Injury Research Center (SCoBIRC)

Traumatic brain injury (TBI) is among the most common causes of acquired temporal lobe epilepsy (TLE). The latent period after head injury and prior to the expression of seizures includes plasticity events that support epileptogenesis, including cell loss and synaptic reorganization in the dentate gyrus. A murine model of TBI using controlled cortical impact (CCI) was used to examine aspects of hilar GABA systems days after injury and the effect of JAK/STAT inhibition on those systems. Western blots were used to assess expression levels of pStat3 as an indicator of JAK/STAT activation in CD-1 mice at 24 hours after CCI or in sham-operated controls. The effectiveness of the Stat3 inhibitor WP1066 (EMD Millipore; 50mg/kg; i.p.; 30 and 90 min post-CCI) on blocking JAK/STAT activation was also tested. The number of surviving GABAergic hilar interneurons was assessed in mice that express GFP in a subset of inhibitory neurons (GIN mice; FVB-Tg(GadGFP)4570Swn/J) 48-72 hours after CCI or in controls. Preliminary results show that CCI results in a dramatic increase in pStat3 protein expression within the injured hemisphere. Low expression of pStat3 was detected in the uninjured hemisphere after CCI, equivalent to that detected in sham-operated controls. Administration of the WP1066 after CCI reduced pStat3 expression to levels similar to those observed in control mice**.** Hilar inhibitory interneurons were reduced in number ipsilateral to the injury in the dorsal hippocampus. Ongoing studies plan to assess the effects of WP1066 on GABAergic hilar interneuron cell loss and how inhibitory synaptic transmission is affected in dentate granule cells during these early time-points following CCI.

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